PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In re Application of:	)	
	)	
Daniel B. Drachman	)	Group Art Unit: 1632
	)	
Serial No. 09/205,096	)	Examiner: E. Sorbello
	)	
Filed: December 3, 1998	)	Atty. Dkt. No. 01107.77737

For: **TARGETING ANTIGEN-SPECIFIC T CELLS FOR SPECIFIC IMMUNOTHERAPY OF AUTOIMMUNE DISEASE**

BRIEF ON APPEAL

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BRIEF ON APPEAL

Assistant Director for Patents
Washington, D.C. 20231

Sir:

An original and two copies of this brief are submitted. Please charge the $160.00 fee for filing the brief and the five-month extension of time fee to our Deposit Account No. 19-0733. Appellants filed the Notice of Appeal on December 13, 2001. If any additional fee is required, please charge our Deposit Account No. 19-0733.

REAL PARTIES IN INTEREST

The real party in interest in this application is The Johns Hopkins University, to which this application is assigned.

RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences.

STATUS OF CLAIMS

Claims 1-40 are canceled. Claims 41-67 stand rejected. Claims 41-67 are appealed.

STATUS OF AMENDMENTS

Claims 54 and 58 were amended in an Amendment After Final Rejection filed October 15, 2001. An Advisory Action mailed November 16, 2001 indicated that the amendment would be entered. The appealed claims are shown in Appendix I.

SUMMARY OF THE INVENTION

One embodiment of the invention is a virus which infects human APCs. The virus comprises a polynucleotide which encodes all or a portion of an auto-antigen to which an auto-immune disease patient's antigen-specific T cells respond. Page 2, lines 11-13.

Another embodiment of the invention is antigen presenting cells of an auto-immune disease patient. The antigen presenting cells are transduced or transfected with a polynucleotide encoding a protein comprising all or a portion of an auto-antigen to which the patient's antigen-specific T cells respond. The all or a portion of an auto-antigen is functionally connected to a signal peptide and a transmembrane/cytoplasmic tail, whereby the all or a portion of auto-antigen is processed by endosomes. Page 3, lines 2-9 and page 3, lines 12-16.

A further embodiment of the invention is a method of ablating auto-antigen-specific T

cell in an auto-immune disease patient. Antigen presenting cells are removed from an auto-immune disease patient. A polynucleotide that encodes all or a portion of an auto-antigen to which the patient's antigen-specific T cells respond is transferred into the APCs. The APCs are reintroduced into the patient, whereby auto-antigen-specific T cells are activated. (Page 2, line 17 to page 3, line 1 and page 3, lines 12-16.) A product which is detrimental to activated T cell proliferation in the patient is administered. (Page 7, lines 8-9.)

ISSUES

I. *The specification discloses an operable method for using the claimed antigen presenting cells and viruses in a model system to test drugs.*

II. *The specification and general knowledge in the art answer all the "how to make and use" questions raised by the Patent Office.*

   a. *Where and how the DNA encoding auto antigens are obtained.*

   b. *How sufficient antigen presenting cells are obtained to practice the method.*

   c. *How the antigen presenting cells are administered to a patient.*

   d. *What detrimental products include.*

   e. *How many cells are administered.*

III. *The generic evidence regarding gene therapy provided by the Patent Office to make a* prima facie *case of non-enablement is not relevant to the claimed methods which employ antigen presenting cells.*

IV. *The Patent Office speculates that the claimed methods, antigen presenting cells, and viruses are not enabled but provides no sound scientific reasoning or evidence to support its assertions.*

   a. *Whether all detrimental products have the same effect on activated T cells.*

   b. *Whether the antigen presenting cells stimulate antigen specific T cells.*

c. *Whether the viral vectors should be modified.*

d. *Whether the antigen presenting cells will work in a patient.*

GROUPING OF CLAIMS

I.	Claims 54-64, 66, and 67 stand or fall separately from claims 41-53 and 65 with respect to issue *I.*
II(a).	Claims 41-67 stand or fall together with respect to issue *II(a).*
II(b) and (c).	Claims 41-53 and 65 stand or fall together with respect to issue *II(b) and (c).* Claims 54-64, 66, and 67 are separate and do not stand or fall with claims 41-53 and 65 with respect to issue *II(b) and (c).*
II(d).	Claims 41, 42, 48-51, 55, 61, and 65 stand or fall separately from claims 43-47, 52-54, 56-60, 62-64, 66, and 67 with respect to issue *II(d).*
II(e).	Claims 41-53 and 65 stand or fall separately from claims 54-64, 66, and 67 with respect to issue *II(e).*
III.	Claims 41-53 and 65 stand or fall separately from claims 54-64, 66, and 67 with respect to issue *III.*
IV(a).	Claims 41, 42, 48-51, 55, 61, and 65 stand or fall separately from claims 43-47, 52-54, 56-60, 62-64, 66, and 67 with respect to issue *IV(a).*
IV(b).	Claims 41- 67 stand or fall together with respect to issue *IV(b).*
IV(c).	Claims 41-48, 50-53, and 65 stand or fall separately from claims 49, 54-64, 66, and 67 with respect to issue *IV(c).*
IV(d).	Claims 41-53 and 65 stand or fall separately from claims 54-64, 66, and 67 with respect to issue *IV(d).*

ARGUMENT

I. *The specification discloses an operable method for using the claimed antigen*

presenting cells and viruses in a model system to test drugs.

Section 112, first paragraph, of Title 35 of the United States Code requires that a patent specification must teach a person skilled in the relevant art how to make and use the invention claimed.

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most clearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

To establish a *prima facie* case of non-enablement, the Examiner has the initial burden to establish a reasonable basis to question the enablement provided in the specification. *In re Wright*, 999 F.2d 1557, 1562, 27 U.S.P.Q. 2d 1510, 1513 (Fed. Cir. 1993).

"The enablement requirement is met if the description enables any mode of making and using the claimed invention." *Engel Industries, Inc. v. Lockformer Company*, 946 F.2d 1528, 1533, 20 U.S.P.Q. 2d 1300 (Fed. Cir. 1991). The Patent Office expands on Engel's holding by explaining, "If multiple uses for claimed compounds or compositions are disclosed in the application, then an enablement rejection must include an explanation, sufficiently supported by the evidence, why the specification fails to enable each disclosed use. In other words, if any use is enabled when multiple uses are disclosed, the application is enabling for the claimed invention." MPEP § 2164.01(c), emphasis added.

Claims 54-57 and 66, directed to antigen presenting cells, all rely on independent claim 54. Claim 54 recites:

> Antigen presenting cells of an auto-immune disease patient which are transduced or transfected with a polynucleotide encoding a

> protein comprising all or a portion of an auto-antigen to which the patient's antigen-specific T cells respond, said all or a portion of an auto-antigen being functionally connected to a signal peptide and a transmembrane/cytoplasmic tail, whereby said all or a portion of auto-antigen is processed by endosomes.

Claims 58-64 and 67, directed to viruses, all rely on independent claim 58. Claim 58 recites:

> A virus which infects human APCs and which comprises a polynucleotide which encodes all or a portion of an auto-antigen to which an auto-immune disease patient's antigen-specific T cells respond.

The specification discloses multiple uses for the antigen presenting cells. First, the specification discloses that the antigen presenting cells can be used to activate antigen-specific T cells in a myasthenia gravis disease patient. "It is another object of the invention to provide autologous antigen presenting cells which have been engineered to express, process and present an antigen to activate antigen-specific T cells of a myasthenia gravis patient." (Page 2, lines 12-13.)

Second, the specification discloses that the antigen presenting cells are useful to screen and identify drugs and treatments for arresting the growth of antigen-specific T cells or eliminating antigen-specific T cells:

> The present invention is based on the discovery that autologous antigen presenting cells (APCs) can be transfected or transduced to express, process, and properly present an antigen to antigen-specific T cells. Moreover, upon proper presentation, the antigen-specific T cells are activated. Activation of the selected class of antigen-specific T cells permits this class to be distinguished from other T cells and for them to be selectively targeted for ablation.
>
> Such antigen-specific T cell activation provides a model system in which drugs and treatments can be screened to identify those which are effective in arresting growth of or eliminating the

antigen-specific T cells.

Page 5, lines 15-23. Thus the specification discloses two distinct uses for the antigen presenting cells.

The Patent Office has challenged the efficacy of the method of using the claimed antigen presenting cells to treat patients. However, the Patent Office has not challenged the efficacy of using the antigen presenting cells in a model system to identify drugs and treatments that eliminate or arrest the growth of antigen-specific T cells. Not one iota of evidence or reasoning has been provided by the Patent Office to challenge this use. [1] The Patent Office has thus not complied with the procedures set out in the Manual of Patent Examination Procedure: "[A]n enablement rejection must include an explanation, sufficiently supported by the evidence, why the specification fails to enable each disclosed use." MPEP § 2164.01(c), emphasis added. Because the Patent Office has failed to comply with its own guidelines for rendering a rejection for failure to teach how to use, the rejection should be reversed as to the antigen presenting cells of claims 54-57 and 66.

The viruses of claims 58-64 and 67 are taught as being useful to make the antigen presenting cells. "It is still another object of the invention to provide a virus for transferring genes to antigen presenting cells so that they express, process, and present an antigen which will stimulate antigen-specific T cells in a myasthenia gravis patient." Page 2, lines 14-16. Because the antigen presenting cells have a disclosed use which has not been challenged, the viruses also have a derivative disclosed use that has not been challenged. The specification discloses at least

[1] The Advisory Action dated April 17, 2002 states that there is only one intended use for the antigen presenting cells and viruses. (Page 2, lines 25-27.) The Patent Office improperly ignores the clear teaching in the specification regarding drug screening.

one operable method of using the antigen presenting cells and viruses that has not been challenged by the Patent Office.

The Board should reverse the enablement rejection of claims 54-64, 66, and 67 because the Patent Office has failed to make a *prima facie* case of nonenablement regarding the antigen presenting cells and viruses.

Claims 41-53 and 65 are directed to methods of treating patients. The efficacy of these therapeutic methods has been specifically challenged by the Patent Office. Thus claims 41-53 and 65 stand separately from claims 54-64, 66, and 67 with respect to this issue.

II. The specification and general knowledge in the art answer all the "how to make and use" questions raised by the Patent Office.

The enablement requirement assures that an inventor provides sufficient information about the claimed invention that a person of skill in the field of the invention can make and use it without undue experimentation, relying on the patent specification and the knowledge in the art. *Scripps Clinic & Research Foundation v. Genentech, Inc.* 927 F.2d 1565, 18 U.S.P.Q. 2d 1001 (Fed. Cir. 1991). "[A] patent need not teach, and preferably omits, what is well known in the art." *Hybritech Inc. v. Monoclonal Antibodies, Inc.* 802 F.2d 1367, 231 U.S.P.Q. (BNA) 81 (Fed. Cir. 1986). The Patent Office raises five separate "how to make and use" questions in asserting that the claimed methods, antigen presenting cells, and viruses are not enabled. The specification and the knowledge in the art provide the answers to each of these questions.

a. Where and how the DNA encoding auto antigens are obtained.

The Patent Office asserts, "The specification does not teach one where and how to obtain the DNA encoding auto antigens." Final Office Action, page 4, lines 16-18. The specification

discloses that any auto-antigen with a known coding sequence can be used in the invention. "This technique can be used with any auto-immune disease in which the inciting auto-antigen is known and for which the coding sequence is available." Page 6, lines 9-10. The nucleotide sequence of at least eleven auto-antigens were known in the art prior to the December 3, 1997 effective filing date. See Appendices 2-12. The eleven nucleotide sequences of human auto-antigens were retrieved from the National Center for Biotechnology Information (NCBI) database. Each was made of record on October 15, 2001. The eleven sequences are listed below and shown as appendices.

- Accession number U01882 discloses the sequence for the human SS-A/Ro autoantigen which is associated with the autoimmune response in mothers whose children have manifestations of neonatal lupus. Appendix 2.

- Accession number D86115 discloses the nucleotide sequence for the human HC21Exc65 exon, an auto antigen in polyglandular disease type I. Appendix 3.

- Accession number X56687 discloses the nucleotide sequence for the human NOR-90 auto antigen, a nucleolar transcription factor that is a novel target for human autoimmune response. Appendix 4.

- Accession number U26593 discloses the nucleotide sequence for the human diabetes mellitus I auto antigen ICAp69. Appendix 5.

- Accession number X54162 discloses the nucleotide sequence for a novel 64-kDa auto antigen recognized patients with autoimmune thyroid disease. Appendix 6.

- Accession number X16163 discloses the nucleotide sequence for a human systemic lupus erythematosus autoimmune antigen. Appendix 7.

- Accession number X69936 discloses the nucleotide sequence for the human glutamic acid decarboxylase auto antigen of insulin-dependent diabetes mellitus patients. Appendix 8.

- Accession number J04977 discloses the nucleotide sequence for the human Ku auto antigen. Appendix 9.

- Accession number J03798 discloses the nucleotide sequence for the human small nuclear ribonucleoprotein Sm-D, an auto antigen of rheumatic disease systemic lupus erythematosus. Appendix 10.

- Accession number M24499 discloses the nucleotide sequence for the human autoantigen cytochrome P450db1, of idiopathic autoimmune hepatitis. Appendix 11.

- Accession number M28639 discloses the nucleotide sequence of human autoimmune thyroid disease-related antigen mRNA. Appendix 12.

The specification also teaches that the acetylcholine receptor is an auto-antigen in myasthenia gravis (MG): "The extracellular portion of the α-subunit (comprising amino acids 1-210) is believed to comprise the epitopes to which most AChR-specific T cells respond." Page 6, lines 18-20. This nucleotide sequence was also known in the art at the time the application was filed and was retrieved from the NCBI database. Accession number M64695 discloses the extracellular portion of AChR α-subunit nucleotide sequence. Appendix 13. The twelve prior art nucleotide sequences of auto-antigens demonstrate that the knowledge in the art was sufficient to enable one of skill in the art to practice the claimed invention without undue experimentation. Claims 41-67 are thus enabled for making the nucleotide sequences of auto-antigens in the invention.

b. How sufficient antigen presenting cells are obtained to practice the method.

The Patent Office inquires "how is one to obtain enough APCs from the patient" to ablate T cells *in vivo*. Final Office Action, page 4, line 20. The combined teachings of the specification and the knowledge in the art teach how to obtain sufficient antigen presenting cells. The specification teaches, "Antigen presenting cells can be withdrawn from an auto-immune disease patient, such as a myasthenia gravis patient according to techniques well known in the

art. They can be found in the blood as well as in the bone marrow. In one embodiment of the invention B cells are purified from the blood and used as the preparation of antigen presenting cells." Page 6, lines 11-15. Furthermore, McLellan, made of record October 15, 2001, teaches a method of isolating a type of antigen presenting cell, a dendritic cell, from blood. *J. Imunol. Methods*, 184, 1995, 81-89, Appendix 14. Combining the teachings in the specification and the knowledge in the art, one of skill in the art would have been able to isolate a sufficient number of antigen presenting cells to practice the claimed invention without undue experimentation. The method claims (41-53 and 65) are thus enabled for obtaining antigen presenting cells.

Claims 54-64, 66, and 67 do not stand with the *ex vivo* method claims because these claims are drawn to antigen presenting cells and viruses that need not be used in a gene therapy method. These cells and viruses can be used to make a model system to screen for drugs and therapeutics as discussed above. Thus they are patentable independent of the *ex vivo* gene therapy issue.

c. When and how the antigen presenting cells are administered to a patient.

The Patent Office asserts, "Applicants did not teach when and how to administer APCs transfected with (1) AchR (AA 1-210), (2) FasL and (3) truncated FADD, to a patient with an auto-immune disease." Final Office Action, page 6, lines 9-11. However, the specification coupled with the knowledge in the art teaches one of skill in the art when and how to administer the antigen presenting cells without undue experimentation.

The specification teaches, "Transduced or transfected autologous antigen presenting cells are re-introduced to the patient using standard techniques for transfusing blood cells." Page 7, lines 5-6. Standard techniques for transfusing blood cells were well known in the art at the time

the application was filed. Goodnough reviews considerations for blood transfusion practices that were known in the art prior to the effective filing date. *AACN Clin. Issues*, 7, 1996, 212-220. Appendix 15. Malech teaches how to transfuse blood cells to an *ex vivo* gene therapy patient. Malech teaches that "transduced PBSCs [peripheral blood stem cells] derived from the apheresis products were administered intravenously." Appendix 16, page 12134, first column, lines 3-4.

The Patent Office has not put forward any reasoning why timing would be critical or would not be within the skill of the ordinary artisan to determine without undue experimentation. The specification and the knowledge in the art provide sufficient information such that one of skill could determine when and how to administer antigen presenting cells to an autoimmune disease patient without undue experimentation. The method claims (41-53 and 65) are enabled for administering the antigen presenting cells.

Claims 54-64, 66, and 67 stand separately from the method claims because these claims are drawn to antigen presenting cells and viruses that need not be used in a gene therapy method. These cells and viruses have an alternative enabled use in a model system to screen for drugs and therapeutics. The cells and viruses need not be administered to a patient. Thus the issue of when and how to administer to a patient does not affect their patentability.

d. What detrimental products include.

The Patent Office asserts that the claims are not enabled because it is unclear "what is the generic 'product' claimed in the instant invention which is detrimental to the activated T cells." Final Office Action, page 4, lines 20-22. The specification, however, clearly teaches one of skill in the art what detrimental products encompass. Detrimental products inactivate or ablate activated antigen specific T cells. Page 7, line 12. In fact, the specification teaches specific

examples of detrimental products. "Detrimental products which have been found to successfully inactivate or ablate activated antigen-specific T cells include CTLA4Ig, a fusion protein which binds to and blocks costimulatory B7 molecules on APC cells, Fas ligand, and antibodies to Fas itself. Antibodies which block costimulatory B7-1 and –2 molecules can also be used." Page 7, lines 12-15. The specification thus names five specific examples of products which are detrimental to activated antigen specific T cells. The specification by means of a generic and specific disclosure adequately teaches the meaning of "detrimental products" that can be used in the method of claims 41, 42, 48-51, and 65, in the antigen presenting cells of claim 55, and in the viruses of claim 61.

This ground of rejection does not apply to the antigen presenting cells of claims 54 and 66, or the viruses of claims 58-60, 64 and 67 because the antigen presenting cells or viruses do not recite a polynucleotide sequence encoding a detrimental product. This ground of rejection also does not apply to method claims 43-47, 52, and 53, antigen presenting cells claims 56 and 57, or virus claims 62 and 63 because all these claims specify that the detrimental product is Fas ligand. The scope of the term "detrimental product" is thus not pertinent to their enablement.

e. How many cells are administered.

The Patent Office asks, "[H]ow is one to know how much is to be administered taking into consideration that the patient has an auto immune disorder and already has activated T cells." Final Office Action, page 4, lines 14-16. One of skill in the art would have known how many cells to administer to a patient receiving *ex vivo* gene therapy due to the level of skill and knowledge generally available.

Malech administered *ex vivo* transduced $CD34^+$ PBSCs for bone marrow to patients with

chronic granulomatous disease. Malech teaches, "The total number of cells infused ranged from 0.1 to 4.7 x 10^6 cells per kg (Table 1)." Appendix 16; page 12135, column 1, lines 5-7; see also pages 12133 to 12134 in the Materials and Methods. One of skill would have referred to such teachings regarding number of cells to administer to an *ex vivo* gene therapy patient. "[I]t is not necessary to specify the dosage or method of use if it is known to one skilled in the art that such information could be obtained without undue experimentation." MPEP § 2164.01(c). Thus method claims 41-53 and 65 could be practiced without undue experimentation.

Claims 54-64, 66, and 67 stand or fall separately from the method claims because these claims are drawn to antigen presenting cells and viruses that need not be used in a gene therapy method. These cells and viruses can be used in an alternative enabled method of screening for drugs and therapeutics. The screening method does not require administration of antigen presenting cells to an autoimmune disease patient.

III. The generic evidence regarding gene therapy provided by the Patent Office to make a prima facie *case of non-enablement is not relevant to the claimed methods which employ antigen presenting cells.*

It is incumbent upon the Patent Office whenever an enablement rejection is made, to explain why it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement. *In re Marzocchi*, 439 F.2d 220, 224, 169 U.S.P.Q. (BNA) 367 (CCPA 1971). The examiner has the initial burden to establish a reasonable basis to question the enablement provided for the claimed invention. *In re Wright*, 999 F.2d 1557, 1562, 27 U.S.P.Q. 2d 1510 (Fed. Cir. 1993).

The Patent Office has stated that the claims are not enabled because "[t]he state of the art

in gene therapy is still in its infancy and is highly unpredictable." Office Action mailed May 25, 2000, page 5, lines 5-6. To support its position, the Patent Office cites three review articles: Orkin (Report and recommendation of the panel to assess the NIH investment in research on gene therapy, 1995), Verma (*Nature* 389, 1997, 239-242), and Crystal (*Science*, 270, 1995, 404-410). Orkin is cited as teaching "Clinical efficacy has not been definitively demonstrated at this time in any gene therapy protocol." Office Action mailed May 25, 2000, page 5, lines 6-7. Verma is cited as teaching that "there is still no single outcome to point to as a success story after hundreds of clinical trials have been performed worldwide on thousands of individuals." Office Action mailed May 25, 2000, page 5, lines 12-13. Crystal is cited as teaching that "there is a significant variation that exists in the genetically marked cells recovered from recipients in *ex vivo* studies." Office Action dated December 13, 2000, page 3, lines 11-13.

None of Orkin, Verma, and Crystal is relevant to the claimed invention because none of these references teaches any example of gene therapy in which polynucleotides were transferred to an antigen presenting cell. Independent claim 41 requires that a polynucleotide which encodes all or a portion of an auto-antigen be transferred into an antigen presenting cell, and that the antigen presenting cell be reintroduced into the patient. Orkin reports on *ex vivo* gene therapy in animal models and in human clinical trials in which nucleic acids are delivered to bone marrow (page 9, paragraph 4), cord blood (page 13, paragraph 4), and liver cells (page 20, paragraph 1). Verma reviews outcomes of animals treated with *ex vivo* gene therapy in which polynucleotides were transferred to mouse primary fibroblasts and myoblasts (page 240, second column, lines 4-12), and to dog myoblasts (page 240, third column, lines 2-9). Verma also reports the outcome of an *ex vivo* human clinical trial in which polynucleotides were transferred to blood progenitor

cells. Page 242, first and second columns. Crystal provides examples of *ex vivo* gene therapy clinical trials in which DNA was transferred to hepatocytes, fibroblasts, and neuroblastoma cells. See Table 2. Crystal also teaches *ex vivo* gene therapy in which polynucleotides were transferred to tumor infiltrating lymphocytes and tumor cells. Page 405, column 3, lines 26-34. There is no teaching, however, of any findings of any *ex vivo* gene therapy clinical trial or animal model in which polynucleotides were transferred to antigen presenting cells.

Appellant has provided specific *in vitro* and *in vivo* evidence in support of the claimed methods of transferring polynucleotides to antigen presenting cells and reintroducing them to an animal. The specification discloses *in vitro* evidence in which a polynucleotide encoding the acetylcholine receptor (AChR) was transferred to antigen presenting cells (A20 cells). Page 12, line 17 to page 13, line 4. The cells were then mixed with AChR-specific T cells. Page 13, lines 22-23. AChR-specific T cells were stimulated by the A20 antigen presenting cells. "Dose response curves indicated that 200 to 1,000 transfected A20 cells maximally stimulate 2.5 x 10^3 AChR-specific T cells in culture." Page 14, lines 18-19. Administration of a detrimental product then ablated the AChR specific T cells. Administration of "CTLA4Ig resulted in 70% inhibition of stimulation." Page 15, line 11. The specification also teaches, "Antibody to Fas resulted in virtually 100% inhibition of T cell proliferation, and treatment with soluble hFasL produced 70 to >90% inhibition." Page 15, lines 18-19. Thus, record evidence demonstrates that antigen specific T cells are activated and ablated by antigen presenting cells that have been modified by transfer of polynucleotides encoding an antigen and a product detrimental to activated T cells.

Appellant's Declaration under Rule 132, executed April 24, 2001, further establishes the

enablement of the claimed methods. Appendix 17. The declaration provides additional *in vitro* evidence that supports the claimed methods. The declaration describes two separate methods. In one, a polynucleotide encoding AChR, a detrimental product (FasL), and truncated FADD was transferred to antigen presenting cells with a vaccinia virus vector. Paragraph 3. Mouse T cell lines specific for AChR were incubated with the transduced antigen presenting cells and were effectively killed. Paragraph 4. The Declaration also described an experiment in which a polynucleotide encoding influenza hemagglutin (HA), FasL, and truncated FADD was transferred to antigen presenting cells with a vaccinia virus vector. Paragraph 5. The antigen presenting cells were incubated with HA-specific T cells and inhibited proliferation of HA specific T cells by approximately one half. Paragraph 5. Thus, the declaration provides further *in vitro* evidence that demonstrates that transfer of polynucleotides to antigen presenting cells is operative.

Appellant's Declaration under Rule 132, executed January 3, 2002, demonstrates that isolated antigen presenting cells (APCs) transduced with a recombinant viral vector encoding an antigen and reintroduced into a mouse ablated antigen-specific T cells. Appendix 18. APCs were removed from an HA transgenic mouse[2] and infected with a recombinant vaccinia virus vector that contained the genes encoding HA, FasL, and truncated FADD, thereby transducing the genes into the APCs. Paragraph 4. The transduced APCs were reintroduced into the HA transgenic mouse. Two days following re-introduction, the percentage of HA-specific $CD4^+$ T cells in the total $CD4^+$ T cell population was 9.4% in mice that received the transduced APCs and 48.8% in the untreated control mice. Paragraph 7. Proliferation of HA-specific splenocytes

[2] The HA transgenic mouse expresses HA-specific T cells.

was reduced by 3.0-3.6 fold in the mouse injected with the modified APCs compared to the mouse that did not received transduced APCs. Similarly, proliferation of HA-specific lymph node cells was approximately 2.4-2.7 fold lower in the mouse with the modified APCs compared to the mouse that did not receive APCs. Paragraph 9. The January 3, 2002 declaration thus clearly demonstrates that activating a particular antigen-specific T cell population with APCs which express the particular antigen, and administering a detrimental product to the activated population successfully ablates antigen-specific T cells *in vivo*.

Appellant has provided in its two declarations specific *in vitro* and *in vivo* evidence which prove that activated antigen-specific T cells are ablated in the presence of a product detrimental to activated T cell proliferation. The evidence further proves that antigen presenting cells (APCs) expressing a particular antigen activate T cells specific for that antigen. The Patent Office has provided no specific evidence to demonstrate that such a method will not work. Claims 41-53 and 65, directed to methods of ablating auto-antigen-specific T cells, are thus enabled.

Claims 54-64, 66, and 67 stand separately from the method claims because these claims are drawn to antigen presenting cells and viruses that need not be used in a gene therapy method. These cells and viruses have an alternative enabled use in a model system to screen for drugs and therapeutics.

IV. The Patent Office speculates that the claimed methods, antigen presenting cells, and viruses are not enabled but provides no sound scientific reasoning or evidence to support its assertions.

It is incumbent upon the Patent Office whenever an enablement rejection is made, to explain why it doubts the truth or accuracy of any statement in a supporting disclosure and to

back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement. *In re Marzocchi*, 439 F.2d 220, 224, 169 U.S.P.Q. (BNA) 367 (CCPA 1971). The Patent Office asserts four speculative grounds why the claims are not enabled. None are supported by any evidence or reasoning.

a. Whether all detrimental products have the same effect on activated T cells.

The Patent Office questions whether "the one example (FasL) used in the in vitro experiments as a product that is detrimental to activated T cells [is] representative of all products detrimental to activated T cells as claimed?" Final Office Action, page 6, lines 14-16. No evidence or sound scientific reasoning has been offered by the Patent Office to doubt appellant's disclosure that the detrimental products will have their intended effect. In any event, Appellant's disclosure teaches that three different detrimental products exert an inhibitory effect on activated AChR specific T cells. The specification teaches that "stimulation by transfected A20 cells [a line of antigen presenting cells that has been transfected with a polynucleotide that encodes AChR] in the presence of 50 μg/ml of CTLA4Ig resulted in 70% inhibition of stimulation." Page 15, lines 10-11. The specification also discloses, "Antibody to Fas resulted in virtually 100% inhibition of T cell proliferation, and treatment with soluble hFasL produced 70 to >90% inhibition." Page 15, lines 18-19. The specification thus affirmatively teaches that three detrimental products, CTLA4Ig, Fas ligand, and antibody to Fas, have an inhibitory effect on activated T cells. The Patent Office has offered no evidence or scientific reasoning to doubt these presumptively true statements. This ground of the rejection should be overturned with regard to claims that recite "detrimental product," *i.e.*, claims 41, 42, 48-51, 55, 61, and 65.

This ground of rejection does not apply to the antigen presenting cells of claims 54 and

66, or the viruses of claims 58-60, 64 and 67 because they do not recite a polynucleotide sequence encoding a detrimental product. This ground of rejection also does not apply to method claims 43-47, 52, and 53, antigen presenting cells claims 56 and 57, or virus claims 62 and 63 because each of these claims specifies that the detrimental product is Fas ligand. The Patent Office tacitly acknowledges that FasL ablates activated T cell proliferation by its very question.

b. Whether the antigen presenting cells stimulate antigen specific T cells.

The Patent Office speculates that "it is not clear that the APCs transfected with AchR, will inactivate the AchR specific T cells rather than stimulate them." Final Office Action, page 4, lines 13-14. The Patent Office appears to have misunderstood the claimed methods. In fact, the claimed methods require that the antigen presenting cells will stimulate antigen specific T cells. First, the antigen-specific T cells are specifically stimulated (activated). Second, a product detrimental to activated T cells is added to ablate them. The claims recite "reintroducing the APCs into the patient, whereby auto-antigen-specific T cells are activated; and administering a product which is detrimental to activated T cell proliferation." It is the activation of the antigen-specific T cells that renders them susceptible to ablation with the detrimental product.

Moreover, the specification discloses that the detrimental products do successfully inactivate the T cells: "When stimulated in the presence of CTLA4Ig, the T cells are relatively inactivated; when stimulated in the presence of Fas ligand or antibody to Fas they undergo apoptosis and death." Page 9, lines 9-11. The Patent Office has offered no evidence or sound scientific reasoning to doubt these statements. There is no reason to doubt appellant's disclosure. This ground of rejection to method claims 41-67 should be overturned.

c. Whether the viral vectors should be modified.

The Patent Office states that upon "introduction of a viral vector [into a patient], unless completely gutted, [the patient] will respond by eliciting an immune response opposing and contrary to the intent of the instant application." Final Office Action, page 5, line 22 through page 6, line 2. The Patent Office offers no evidence or sound scientific reasoning to support its assertion that the viral vector will elicit any immune response that is contrary to the intent of the application. Moreover, the Patent Office provides no evidence that such a response if elicited would prevent the successful practice of the invention. Thus this ground of rejection should be overturned for failure to make a *prima facie* case with respect to claims 41-48, 50-53, and 65 which are directed to methods of ablating auto-antigen-specific T cells in an auto-immune disease patient.

Claims 54-64, 66, and 67 are separately patentable with respect to this ground because these claims are drawn to antigen presenting cells and viruses that can be used in a model system to screen for drugs and therapeutics. No immune response would be elicited in such an *in vitro* model system. This ground of rejection also does not apply to claim 49. Claims 49 specifies that the virus is attenuated, so it would not evoke an immune response.

d. Whether the antigen presenting cells will work in a patient.

The Patent Office speculates that the antigen presenting cells will not have the same effect in an autoimmune disease patient as they do in *in vitro* experiments. This ground applies only to method claims 41-53 and 65. The Patent Office speculates that "an APC or any cell that reacts in a specific manner in a cell culture environment may not react in a similar manner when placed in a patient's body." Final Office Action, page 4, lines 7-8. The Patent Office also

questions whether "the APC cells removed from the patient and transfected with a polynucleotide encoding at least a portion of AChR and FasL and FADD will simulate that which occurs in a lymphoma cell line transfected ex vivo." Final Office Action, page 4, lines 10-13. The Patent Office, however, provides no evidence or sound scientific reasoning that contradicts appellant's teachings that the methods will work as claimed. As discussed above, appellant's declaration executed January 3, 2002, supports the operability of the claimed methods *in vivo*. The declaration teaches that isolated antigen presenting cells (APCs) transduced with a recombinant viral vector containing a gene encoding an antigen (HA), a detrimental product, and truncated FADD, kills T cells that recognize the HA antigen in an animal. This is probative evidence that the antigen presenting cells will function *in vivo* in a patient.

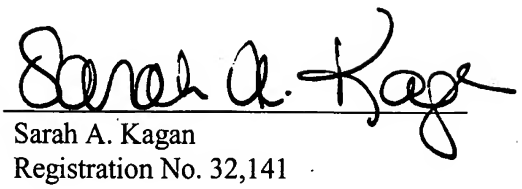
Claims 54-64, 66, and 67 are separately patentable with respect to this ground because these claims are drawn to antigen presenting cells and viruses that need not be used in patients but can be used in an enabled model system to screen for drugs and therapeutics.

CONCLUSION

For the reasons given above, the rejection of claims 41-67 under 35 U.S.C. § 112, first paragraph, is improper. The Board of Patent Appeals and Interferences should reverse the rejection.

Respectfully submitted,

Date: July 15, 2002

By: 

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APPENDIX I. APPEALED CLAIMS

41. A method of ablating auto-antigen-specific T cells in an auto-immune disease patient comprising the steps of:

removing antigen presenting cells (APCs) from an auto-immune disease patient;

transferring into the APCs a polynucleotide which encodes all or a portion of an auto-antigen to which the patient's antigen-specific T cells respond;

reintroducing the APCs into the patient, whereby auto-antigen-specific T cells are activated; and

administering a product which is detrimental to activated T cell proliferation in the patient.

42. The method of claim 41 wherein the polynucleotide which encodes all or a portion of an auto-antigen further encodes a signal sequence and a transmembrane/cytoplasmic tail, said signal sequence and transmembrane/cytoplasmic tail being functionally located with respect to the auto-antigen or portion thereof to facilitate the auto-antigen's endosomal processing.

43. The method of claim 41 wherein the product is Fas ligand.

44. The method of claim 43 wherein the Fas ligand is administered by administration of APC cells which express Fas ligand.

45. The method of claim 43 wherein the APC cells which express Fas ligand also express a truncated form of FADD which is sufficient to protect a cell also expressing Fas from apoptosis.

46. The method of claim 45 wherein the APC cells which express FAS ligand and a truncated

form of FADD are the same cells which express auto-antigen.

47. The method of claim 44 wherein the APC cells which express FAS ligand are the same cells which express auto-antigen.

48. The method of claim 41 wherein the polynucleotides is a viral genome.

49. The method of claim 48 wherein the viral genome encodes an attenuated virus.

50. The method of claim 48 wherein the viral genome is a Vaccinia virus genome.

51. The method of claim 48 wherein said polynucleotide further encodes a product which is detrimental to activated T cell proliferation.

52. The method of claim 51 wherein the product is Fas ligand.

53. The method of claim 52 wherein the polynucleotide further encodes a truncated form of FADD which is sufficient to protect a cell also expressing Fas from apoptosis.

54. Antigen presenting cells of an auto-immune disease patient which are transduced or transfected with a polynucleotide encoding a protein comprising all or a portion of an auto-antigen to which the patient's antigen-specific T cells respond, said all or a portion of an auto-antigen being functionally connected to a signal peptide and a transmembrane/cytoplasmic tail, whereby said all or a portion of auto-antigen is processed by endosomes.

55. The antigen presenting cells of claim 54 which are transduced or transfected with a polynucleotide sequence encoding a protein which is detrimental to activated T cell survival.

56. The antigen presenting cells of claim 55 wherein the protein which is detrimental to activated T cell survival is Fas ligand.

57. The antigen presenting cells of claim 56 which have been transduced or transfected with a polynucleotide sequence encoding a truncated form of FADD which is sufficient to protect a cell also expressing Fas from apoptosis.

58. A virus which infects human APCs and which comprises a polynucleotide which encodes all or a portion of an auto-antigen to which an auto-immune disease patient's antigen-specific T cells respond.

59. The virus of claim 58 which is a Vaccinia virus.

60. The virus of claim 58, wherein the polynucleotide further encodes a signal sequence and a transmembrane/cytoplasmic tail both of which are functionally connected to said all or portion of auto antigen, whereby the encoded all or a portion of auto-antigen is processed by endosomes.

61. The virus of claim 60 further comprising a nucleotide sequence which encodes a product detrimental to proliferation of activated T cells.

62. The virus of claim 61 wherein the product is Fas ligand.

63. The virus of claim 62 further comprising a nucleotide sequence which encodes a truncated form of FADD which is sufficient to protect a cell also expressing Fas from apoptosis.

64. The virus of claim 58 which is attenuated.

65. The method of claim 41 wherein the auto-antigen is an extracellular domain of α-subunit of acetylcholine receptor and the auto-immune disease is *myasthenia gravis*.

66. The antigen presenting cells of claim 54 wherein the auto-antigen is an extracellular domain of α-subunit of acetylcholine receptor and the auto-immune disease is *myasthenia gravis*.

67. The virus of claim 58 wherein the auto-antigen is an extracellular domain of α-subunit of acetylcholine receptor and the auto-immune disease is *myasthenia gravis*.

APPENDIX 2

☐ 1: U01882. Homo sapiens SS-A...[gi:499209] Related Sequences, OMIM, Protein, PubMed, Taxonomy, LinkOut

```
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VERSION     U01882.1  GI:499209
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REFERENCE   1  (bases 1 to 13270)
  AUTHORS   Chan,E.K., Hamel,J.C., Buyon,J.P. and Tan,E.M.
  TITLE     Molecular definition and sequence motifs of the 52-kD component of
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  JOURNAL   J. Clin. Invest. 87 (1), 68-76 (1991)
  MEDLINE   91086480
REFERENCE   2  (bases 1 to 13270)
  AUTHORS   Chan,E.K., Di Donato,F., Hamel,J.C., Tseng,C.E. and Buyon,J.P.
  TITLE     52-kD SS-A/Ro: genomic structure and identification of an
            alternatively spliced transcript encoding a novel leucine
            zipper-minus autoantigen expressed in fetal and adult heart
  JOURNAL   J. Exp. Med. 182 (4), 983-992 (1995)
  MEDLINE   96018798
REFERENCE   3  (bases 1 to 13270)
  AUTHORS   Chan,E.K.
  TITLE     Direct Submission
  JOURNAL   Submitted (16-SEP-1993) Edward K. Chan, Molecular and Experimental
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APPENDIX 3

☐ 1: D86115. Homo sapiens gene...[gi:2244724] OMIM, PubMed, Taxonomy

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LOCUS       D86115          90 bp    DNA              PRI       04-JUL-1997
DEFINITION  Homo sapiens gene for HC21EXc65, exon.
ACCESSION   D86115
VERSION     D86115.1  GI:2244724
KEYWORDS    HC21EXc65.
SOURCE      Homo sapiens B-lymphoblastoid cell_line:GM130B DNA,
            clone_lib:chromosome 21-specific cosmid library.
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 90)
  AUTHORS   Shimizu,N.
  TITLE     Direct Submission
  JOURNAL   Submitted (19-JUN-1996) Nobuyoshi Shimizu, Keio University School
            of Medicine, Department of Molecular Biology; 35 Shinanomachi,
            Shinjuku-ku, Tokyo 160, Japan (E-mail:shimizu@dmb.med.keio.ac.jp,
            Tel:03-3351-2370, Fax:03-3351-2370)
REFERENCE   2  (sites)
  AUTHORS   Nagamine,K., Kudoh,J., Asakawa,S., Abe,I., Maeda,H., Tsujimoto,S.,
            Minoshima,S., Ito,F. and Shimizu,N.
  TITLE     Localization of 22 Exons to a 450-kb Region Involved in the
            Autoimmune Polyglandular Disease Type I (APECED) on Choromosome
            21q22.3
  JOURNAL   Unpublished
REFERENCE   3  (sites)
  AUTHORS   Kudoh,J., Nagamine,K., Asakawa,S., Abe,I., Kawasaki,K., Maeda,H.,
            Tsujimoto,S., Minoshima,S., Ito,F. and Shimizu,N.
  TITLE     Localization of 16 exons to a 450-kb region involved in the
            autoimmune polyglandular disease type I (APECED) on human
            chromosome 21q22.3
  JOURNAL   DNA Res. 4 (1), 45-52 (1997).
  MEDLINE   97323005
FEATURES             Location/Qualifiers
     source          1..90
                     /organism="Homo sapiens"
                     /db_xref="taxon:9606"
                     /chromosome="21"
                     /map="21q22.3"
                     /cell_line="GM130B"
                     /cell_type="B-lymphoblastoid"
                     /clone_lib="chromosome 21-specific cosmid library"
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     exon            1..90
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APPENDIX 4

☐ 1: X56687. H.sapiens mRNA fo...[gi:28970] Related Sequences, OMIM, Protein, PubMed, Taxonomy, LinkOut

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LOCUS       HSAUTNOR     2489 bp    mRNA            PRI       03-APR-1997
DEFINITION  H.sapiens mRNA for autoantigen NOR-90.
ACCESSION   X56687
VERSION     X56687.1  GI:28970
KEYWORDS    NOR-90 autoantigen.
SOURCE      human.
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 2489)
  AUTHORS   Chan,E.K.L.
  TITLE     Direct Submission
  JOURNAL   Submitted (15-NOV-1990) Chan E.K.L., Scripps Clinic & Research
            Foundation, 10666 N Torrey Pines Road, La Jolla CA 92037, U S A
REFERENCE   2  (bases 1 to 2489)
  AUTHORS   Chan,E.K., Imai,H., Hamel,J.C. and Tan,E.M.
  TITLE     Human autoantibody to RNA polymerase I transcription factor hUBF.
            Molecular identity of nucleolus organizer region autoantigen NOR-90
            and ribosomal RNA transcription upstream binding factor
  JOURNAL   J. Exp. Med. 174 (5), 1239-1244 (1991)
  MEDLINE   92044316
FEATURES             Location/Qualifiers
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                     /cell_line="HepG2 and MOLT-4"
                     /cell_type="liver and T cell origin"
                     /clone_lib="gZap, HepG2 cDNA and ggt11 MOLT-4 cDNA"
     CDS             180..2363
                     /codon_start=1
                     /product="autoantigen NOR-90"
                     /protein_id="CAA40016.1"
                     /db_xref="GI:28971"
                     /db_xref="SWISS-PROT:P17480"
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                     PELSESELTRLLARMWNDLSEKKKAKYKAREAALKAQSERKPGGEREERGKLPESPKR
                     AEEIWQQSVIGDYLARFKNDRVKALKAMEMTWNNMEKKEKLMWIKKAAEDQKRYEREL
                     SEMRAPPAATNSSKKMKFQGEPKKPPMNGYQKFSQELLSNGELNHLPLKERMVEIGSR
                     WQRISQSQKEHYKKLAEEQQKQYKVHLDLWVKSLSPQDRAAYKEYISNKRKSMTKLRG
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BASE COUNT 704 a 668 c 740 g 377 t
ORIGIN

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      61 gagccaggga gggagccgct ggagcggaag cccggaggcc gcgctgcgcc ggggtgaggt
     121 ggctttgacc ccgggttgcc cggccagcac gaccgaggag gtggctggac agctggagga
     181 tgaacggaga agccgactgc cccacagacc tggaaatggc cgcccccaaa ggccaagacc
     241 gttggtccca ggaagacatg ctgactttgc tggaatgcat gaagaacaac cttccatcca
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     421 agttccgtac attgacagaa ttgatcctcg atgctcagga acatgttaaa aatccttaca
     481 aaggcaaaaa actcaagaaa cacccagact tcccaaagaa gcccctgacc ccttatttcc
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APPENDIX 5

☐ 1: U26593. Human clone 61.1 ...[gi:1674389] Related Sequences, OMIM, Protein, PubMed, Taxonomy

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LOCUS       HSU26593      592 bp    DNA             PRI       18-NOV-1996
DEFINITION  Human clone 61.1 diabetes mellitus type I autoantigen (ICAp69)
            gene, partial cds.
ACCESSION   U26593
VERSION     U26593.1  GI:1674389
KEYWORDS    .
SOURCE      human.
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 592)
  AUTHORS   Pietropaolo,M., Castano,L., Babu,S., Buelow,R., Kuo,Y.-L.S.,
            Martin,S., Martin,A., Powers,A.C., Prochazka,M., Naggert,J.,
            Leiter,E.H. and Eisenbarth,G.S.
  TITLE     Islet cell autoantigen 69 kD (ICA69). Molecular cloning and
            characterization of a novel diabetes-associated autoantigen
  JOURNAL   J. Clin. Invest. 92 (1), 359-371 (1993)
  MEDLINE   93315668
REFERENCE   2  (bases 1 to 592)
  AUTHORS   Miyazaki,I., Gaedigk,R., Hui,M.F., Cheung,R.K., Morkowski,J.,
            Rajotte,R.V. and Dosch,H.M.
  TITLE     Cloning of human and rat p69 cDNA, a candidate autoimmune target in
            type 1 diabetes
  JOURNAL   Biochim. Biophys. Acta 1227 (1-2), 101-104 (1994)
  MEDLINE   95002197
REFERENCE   3  (bases 1 to 592)
  AUTHORS   Gaedigk,R., Duncan,A.M., Miyazaki,I., Robinson,B.H. and Dosch,H.M.
  TITLE     ICA1 encoding p69, a protein linked to the development of type 1
            diabetes, maps to human chromosome 7p22
  JOURNAL   Cytogenet. Cell Genet. 66 (4), 274-276 (1994)
  MEDLINE   94215321
REFERENCE   4  (bases 1 to 592)
  AUTHORS   Miyazaki,I., Cheung,R.K., Gaedigk,R., Hui,M.F., Van der Meulen,J.,
            Rajotte,R.V. and Dosch,H.M.
  TITLE     T cell activation and anergy to islet cell antigen in type I
            diabetes
  JOURNAL   J. Immunol. 154 (3), 1461-1469 (1995)
  MEDLINE   95123099
REFERENCE   5  (bases 1 to 592)
  AUTHORS   Karges,W.J.P., Gaedigk,R., Hui,M.F., Cheung,R.K. and Dosch,H.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (08-MAY-1995) Hans-Michael Dosch, Immunology and Cancer,
            The Hospital for Sick Children, 555 University Ave., Toronto,
            Ontario M5G 1X8, Canada
FEATURES             Location/Qualifiers
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                     /db_xref="taxon:9606"
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     exon            392..488
     gene            472..592
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     CDS             472..>488
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                     /codon_start=1
                     /product="diabetes mellitus type I autoantigen"
                     /protein_id="AAB19035.1"
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                     /translation="MSGHK"
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                     /gene="ICAp69"
BASE COUNT      181 a    101 c     88 g    222 t
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       61 ttcaccttat aatattgtat acctcaacaa catatagtat tgttttacat atttttaagc
      121 tttatataaa tgatattata ttttatgtag tcttctgcaa cctgcttttt cattcaatct
      181 gcctgttcac tttttaaact ttatttggaa tatttcttcc agtgttactt agctatattg
      241 ttgtcatcac cagtgtgcag cattactgtg taacagtgtt agaccctaaa atacagctag
      301 aaatggctta ggttgaaggc ttttatgtgt gtcacattaa tatgtaacta taatatggtg
      361 tgtaactatt aaacatattt gttttatttc aggttataat ataacttatc ctctcatgct
      421 tttttcctgc cccttctccc caaatcatca acaatagaag aagaagaaaa catgtcagga
      481 cacaaatggt aagtaatttg tcatcttttc tgaattggga aagttagaaa gcatatagca
      541 tatgatcaat aaacatctat tgaatggaaa aacaagagat acatttggca gt
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APPENDIX 6

☐ 1: X54162. Human mRNA for a ...[gi:28968] Related Sequences, OMIM, Protein, PubMed, Taxonomy, LinkOut

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LOCUS       HSAUTAN64     3881 bp    mRNA            PRI       25-JUL-1991
DEFINITION  Human mRNA for a 64 Kd autoantigen expressed in thyroid and
            extra-ocular  muscle.
ACCESSION   X54162
VERSION     X54162.1  GI:28968
KEYWORDS    64 Kd autoantigen; autoantigen.
SOURCE      human.
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 3881)
  AUTHORS   Dong,Q.H.
  TITLE     Direct Submission
  JOURNAL   Submitted (30-JUL-1990) Dong Q.H., Institute of Interdisciplinary
            Research, School of Medicine, Universite Libre de Bruxelles, Campus
            Erasme, 808 Route de Lennik, 1070 Brussels, Belgium
REFERENCE   2  (bases 1 to 3881)
  AUTHORS   Dong,Q., Ludgate,M. and Vassart,G.
  TITLE     Cloning and sequencing of a novel 64-kDa autoantigen recognized by
            patients with autoimmune thyroid disease
  JOURNAL   J. Clin. Endocrinol. Metab. 72 (6), 1375-1381 (1991)
  MEDLINE   91225220
FEATURES             Location/Qualifiers
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                     /db_xref="taxon:9606"
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                     /clone_lib="lambda gt11"
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                     EEKKGGDRNTGLSRDKDKKREEMKEVAKKEDDEKVKGERRNTDTRKEGEKMKRAGGNT
                     DMKKEDEKVKRGTGNTDTKKDDEKVKKNEPLHEKEAKDDSKTKTPEKQTPSGPTKPSE
                     GPAKVEEEAAPSIFDEPLERVKNNDPEMTEVNVNNSDCITNEILVRFTEALEFNTVVK
                     LFALANTRADDHVAFAIAIMLKANKTITSLNLDSNHITGKGILAIFRALLQNNTLTEL
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                     LQEQRQAQEAKGEKKDLLEVPKAGAVAKGSPKPSPQPSPKPSPKNSPKKGGAPAAPPP
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BASE COUNT 1065 a 1030 c 1084 g 702 t
ORIGIN

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     181 gacagcctgc tgggaccctg tctcccgagg agatggagga gctggagaag gagctggacg
     241 tggtggaccc agacgggagt gttcccgtgg ggctgcggca gagaaaccag acggagaaac
     301 agtccacggg tgtgtacaac cgggaggcca tgctcaactt ctgtgaaaag gagaccaaga
     361 aacttatgca gagggagatg tccatggatg aaagcaagca agtggagacc aagacagatg
     421 ccaagaatgg acaggaaagg ggcagagatg ccagcaaaaa agccctgggc cccagacgga
     481 actcagatct ggggaaggag ccaaagaggg gtggtttaaa gaaaagcttc tctagagaca
     541 gagatgaagc tggtggcaag agtggcgaga agcccaagga ggagaagatc atccggggca
     601 ttgacaaggg ccgggtcagg gctgcagtgg ataagaagga ggcagggaag gatgggagag
     661 gagaggagag ggcagtggcc accaagaagg aagaggagaa gaaagggggt gacaggaaca
     721 caggcttgag cagggacaag gataaaaaga gagaggagat gaaggaggtg gccaagaaag
     781 aggatgatga gaaggtaaaa ggggagcgta ggaacacaga caccagaaaa gagggtgaga
     841 agatgaaaag agcaggtggg aacacagaca tgaaaaagga ggatgagaag gtaaaaagag
     901 gaactgggaa cacagacacc aaaaaggacg atgaaaaagt caagaagaat gaacccttac
     961 atgaaaagga agccaaggat gacagcaaga ccaaaacacc cgagaaacag acgcccagtg
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    1381 ccgagctccg cttccacaac cagcgacaca tctgtggagg caagacggag atggagatcg
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    1681 agccctctcc aaagaactca cccaaaaaag ggggtgctcc agctgcccca ccacccctc
    1741 cccctccctt ggctccaccc cttatcatgg agaacctgaa gaattcactc tcaccagcta
    1801 cccagaggaa gatgggagac aaagtcctcc ctgcccagga gaagaactcc cgtgaccagc
    1861 tattggctgc catccgctcc agcaacctca agcagctcaa gaaggtggaa gtgcccaaac
    1921 tgcttcagta ggaccaggct gccaggcacc atctgccaat gccatgactg ctcaggcctc
    1981 acctcccagg gctacacaga ccctgcccac cccatccctg gctgacctgc tgtggatgtc
    2041 cctattctgc catgggagcg tccaggcctg ggtcacgctc aaggaaggat gccttatctc
    2101 ttctcacttt ccttttcttg tctctgaggc tctccaaatt ttgctttagt acatggagct
    2161 caggtttctg gacaagaaga gtccttttag cacatcactg agaagatggc actgtccagg
    2221 gcccatgtag ctggcaagct gcaaaaggcc tgtgatccag gaaagatgtc ccacagggac
    2281 cacatccacc ccagccccac tgccctccag ggccaggatt caggcctctg aggagcccac
    2341 ggggcaaagc tgctgggcca gtggcactct gtgtgggaaa atggcagaaa gatggagagg
    2401 catgggggcc caaaggggag cgtggggagg ggctgaggat accccaaagt ccaggctaat
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    2521 acaggaggag tgggacagac agcagctgga cttgaaggtt tgatgccaaa gcagacattt
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    2881 tctactaggt ctgctcctga accagtcctg ctgcctggag tcagtagcca gagttgttct
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    3301 cactgtggaa ctgccacctt gggtctgtgt cacttgtagg tttctctgcc tccaggttgc
    3361 ctcaacagca ggaggcacag cagtttcacc atctttgagg tgagggtggg gtgccccagc
    3421 taggaagcaa gatcgctgtg ctaggtctga ccaaaaccag agggcagtct agtcctgggg
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3661 ctgtcagccc cactgcaccc ccctgtgcct gctgggcact gggcactagc tagatgcttt
3721 aggttgcttc agctgatcct tcaactctgt gaggtggata ccaatattct attttgcaga
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APPENDIX 7

1: X16163. Human mRNA for Sm... [gi:36494]

Related Sequences, OMIM, Protein, PubMed, Taxonomy, LinkOut

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LOCUS       HSSMB          713 bp    mRNA            PRI       19-JUL-1995
DEFINITION  Human mRNA for SmB/B' autoimmune antigen (K5-1).
ACCESSION   X16163
VERSION     X16163.1  GI:36494
KEYWORDS    autoimmune antigen; systemic lupus erythematosus.
SOURCE      human.
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 713)
  AUTHORS   Sharpe,N.G., Williams,D.G., Howarth,D.N., Coles,B. and
            Latchman,D.S.
  TITLE     Isolation of cDNA clones encoding the human Sm B/B' auto-immune
            antigen and specifically reacting with human anti-Sm auto-immune
            sera
  JOURNAL   FEBS Lett. 250 (2), 585-590 (1989)
  MEDLINE   89325637
FEATURES             Location/Qualifiers
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     mRNA            1..713
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                     /evidence=experimental
     CDS             <1..657
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                     /db_xref="GI:36495"
                     /translation="DGRIFIGTFKAFDKHMNLILCDCDEFRKIKPKNAKQPEREEKRV
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APPENDIX 8


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LOCUS       HSGLAD2A     1803 bp    mRNA            PRI       16-MAR-1993
DEFINITION  H.sapiens mRNA for glutamate decarboxylase.
ACCESSION   X69936
VERSION     X69936.1  GI:31757
KEYWORDS    autoantigen; glutamate decarboxylase; glutamic acid decarboxylase;
            histidine-hexapeptide fusion protein.
SOURCE      human.
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 1803)
  AUTHORS   Northemann,W.
  TITLE     Direct Submission
  JOURNAL   Submitted (23-DEC-1992) W. Northemann, Elias Entwicklungslabor,
            Department of molecular Biology, Obere Hardtstrasse 18, D-7800
            Freiburg, FRG
REFERENCE   2  (bases 1 to 1803)
  AUTHORS   Mauch,L., Abney,C.C., Berg,H., Scherbaum,W.A., Liedvogel,B. and
            Northemann,W.
  TITLE     Characterization of a linear epitope within the human pancreatic
            64-kDa glutamic acid decarboxylase and its autoimmune recognition
            by sera from insulin-dependent diabetes mellitus patients
  JOURNAL   Eur. J. Biochem. 212 (2), 597-603 (1993)
  MEDLINE   93185681
FEATURES             Location/Qualifiers
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     CDS             1..1803
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                     NWELADQPQNLEEILMHCQTTLKYAIKTGHPRYFNQLSTGLDMVGLAADWLTSTANTN
                     MFTYEIAPVFVLLEYVTLKKMREIIGWPGGSGDGIFSPGGAISNMYAMMIARFKMFPE
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                     DKVNFFRMVISNPAATHQDIDFLIEEIERLGQDL"
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                     /gene="GAD2"
                     /note="histidine-hexapeptide"
     misc_feature    61..1803
                     /gene="GAD2"
                     /note="glutamate decarboxylase"
BASE COUNT      495 a    404 c    463 g    441 t
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      301 gatgtcaact acgcgtttct ccatgcaaca gacctgctgc cggcgtgtga tggagaaagg
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     1381 tgcggacgcc acgttgatgt ttttaaacta tggctgatgt ggagggcaaa ggggactacc
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     1501 aaaaaccgag aaggatatga gatggtgttt gatgggaagc ctcagcacac aaatgtctgc
     1561 ttctggtaca ttcctccaag cttgcgtact ctggaagaca atgaagagag aatgagtcgc
     1621 ctctcgaagg tggctccagt gattaaagcc agaatgatgg agtatggaac cacaatggtc
     1681 agctaccaac ccttgggaga caaggtcaat ttcttccgca tggtcatctc aaacccagcg
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     1801 taa
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```

APPENDIX 9

Nucleotide

PubMed Nucleotide Protein Genome Structure PopSet Taxonomy OMIM

Search Nucleotide for Go Clear

Limits Preview/Index History Clipboard Details

Display GenBank as HTML Save Add to Clipboard

☐ 1: J04977. Human Ku autoimmu...[gi:186791] Related Sequences, OMIM, Protein, PubMed, Taxonomy, LinkOut

```
LOCUS       HUMKUANT     3052 bp    mRNA            PRI       06-JAN-1995
DEFINITION  Human Ku autoimmune antigen gene, complete cds.
ACCESSION   J04977
VERSION     J04977.1  GI:186791
KEYWORDS    Ku antigen; nonhistone DNA binding protein; nuclear protein.
SOURCE      Human fetal liver, cDNA to mRNA.
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 3052)
  AUTHORS   Yaneva,M., Wen,J., Ayala,A. and Cook,R.
  TITLE     cDNA-derived amino acid sequence of the 86-kDa subunit of the Ku
            antigen
  JOURNAL   J. Biol. Chem. 264 (23), 13407-13411 (1989)
  MEDLINE   89340410
COMMENT     Draft entry and computer-readable sequence for [1] kindly submitted
            by M.Yaneva, 02-JUN-1989.
FEATURES             Location/Qualifiers
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                     /db_xref="taxon:9606"
                     /map="22q11-q13"
     mRNA            1..3052
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     gene            34..2232
                     /gene="G22P1"
     CDS             34..2232
                     /gene="G22P1"
                     /note="Ku antigen"
                     /codon_start=1
                     /protein_id="AAA59475.1"
                     /db_xref="GI:307093"
                     /db_xref="GDB:G00-119-963"
                     /translation="MVRSGNKAAVVLCMDVGFTMSNSIPGIESPFEQAKKVITMFVQR
                     QVFAENKDEIALVLFGTDGTDNPLSGGDQYQNITVHRHLMLPDFDLLEDIESKIQPGS
                     QQADFLDALIVSMDVIQHETIGKKFEKRHIEIFTDLSSRFSKSQLDIIIHSLKKCDIS
                     LQFFLPFSLGKEDGSGDRGDGPFRLGGHGPSFPLKGITEQQKEGLEIVKMVMISLEGE
                     DGLDEIYSFSESLRKLCVFKKIERHSIHWPCRLTIGSNLSIRIAAYKSILQERVKKTW
                     TVVDAKTLKKEDIQKETVYCLNDDDETEVLKEDIIQGFRYGSDIVPFSKVDEEQMKYK
                     SEGKCFSVLGFCKSSQVQRRFFMGNQVLKVFAARDDEAAAVALSSLIHALDDLDMVAI
                     VRYAYDKRANPQVGVAFPHIKHNYECLVYVQLPFMEDLRQYMFSSLKNSKKYAPTEAQ
                     LNAVDALIDSMSLAKKDEKTDTLEDLFPTTKIPNPRFQRLFQCLLHRALHPREPLPPI
                     QQHIWNMLNPPAEVTTKSQIPLSKIKTLFPLIEAKKKDQVTAQEIFQDNHEDGPTAKK
                     LKTEQGGAHFSVSSLAEGSVTSVGSVNPAENFRVLVKQKKASFEEASNQLINHIEQFL
                     DTNETPYFMKSIDCIRAFREEAIKFSEEQRFNNFLKALQEKVEIKQLNHFWEIVVQDG
                     ITLITKEEASGSSVTAEEAKKFLAPKDKPSGDTAAVFEEGGDVDDLLDMI"
BASE COUNT      906 a    592 c    708 g    846 t
ORIGIN      58 bp upstream of PvuII site.
```

```
   1 ggcgggcgac caaagcgcct gaggaccggc aacatggtgc ggtcggggaa taaggcagct
  61 gttgtgctgt gtatggacgt gggctttacc atgagtaact ccattcctgg tatagaatcc
 121 ccatttgaac aagcaaagaa ggtgataacc atgtttgtac agcgacaggt gtttgctgag
 181 aacaaggatg agattgcttt agtcctgttt ggtacagatg gcactgacaa tccccttttct
 241 ggtggggatc agtatcagaa catcacagtg cacagacatc tgatgctacc agattttgat
 301 ttgctggagg acattgaaag caaaatccaa ccaggttctc aacaggctga cttcctggat
 361 gcactaatcg tgagcatgga tgtgattcaa catgaaacaa taggaaagaa gtttgagaag
 421 aggcatattg aaatattcac tgacctcagc agccgattca gcaaaagtca gctggatatt
 481 ataattcata gcttgaagaa atgtgacatc tccctgcaat tcttcttgcc tttctcactt
 541 ggcaaggaag atggaagtgg ggacagagga gatggcccct ttcgcttagg tggccatggg
 601 ccttcctttc cactaaaagg aattaccgaa cagcaaaaag aaggtcttga gatagtgaaa
 661 atggtgatga tatctttaga aggtgaagat gggttggatg aaatttattc attcagtgag
 721 agtctgagaa aactgtgcgt cttcaagaaa attgagaggc attccattca ctggccctgc
 781 cgactgacca ttggctccaa tttgtctata aggattgcag cctataaatc gattctacag
 841 gagagagtta aaaagacttg gacagttgtg gatgcaaaaa ccctaaaaaa agaagatata
 901 caaaaagaaa cagtttattg cttaaatgat gatgatgaaa ctgaagtttt aaaagaggat
 961 attattcaag ggttccgcta tggaagtgat atagttcctt tctctaaagt ggatgaggaa
1021 caaatgaaat ataaatcgga ggggaagtgc ttctctgttt tgggattttg taaatcttct
1081 caggttcaga gaagattctt catgggaaat caagttctaa aggtctttgc agcaagagat
1141 gatgaggcag ctgcagttgc actttcctcc ctgattcatg ctttggatga cttagacatg
1201 gtggccatag ttcgatatgc ttatgacaaa agagctaatc ctcaagtcgg cgtggctttt
1261 cctcatatca agcataacta tgagtgttta gtgtatgtgc agctgccttt catggaagac
1321 ttgcggcaat acatgttttc atccttgaaa aacagtaaga aatatgctcc caccgaggca
1381 cagttgaatg ctgttgatgc tttgattgac tccatgagct tggcaaagaa agatgagaag
1441 acagacaccc ttgaagactt gtttccaacc accaaaatcc caaatcctcg atttcagaga
1501 ttatttcagt gtctgctgca cagagcttta catccccggg agcctctacc cccaattcag
1561 cagcatattt ggaatatgct gaatcctccc gctgaggtga caacgaaaag tcagattcct
1621 ctctctaaaa taaagaccct ttttcctctg attgaagcca agaaaaagga tcaagtgact
1681 gctcaggaaa ttttccaaga caaccatgaa gatggaccta cagctaaaaa attaaagact
1741 gagcaagggg gagcccactt cagcgtctcc agtctggctg aaggcagtgt cacctctgtt
1801 ggaagtgtga atcctgctga aaacttccgt gttctagtga aacagaagaa ggccagcttt
1861 gaggaagcga gtaaccagct cataaatcac atcgaacagt ttttggatac taatgaaaca
1921 ccgtatttta tgaagagcat agactgcatc cgagccttcc gggaagaagc cattaagttt
1981 tcagaagagc agcgctttaa caacttcctg aaagcccttc aagagaaagt ggaaattaaa
2041 caattaaatc atttctggga aattgttgtc caggatggaa ttactctgat caccaaagag
2101 gaagcctctg gaagttctgt cacagctgag gaagccaaaa agtttctggc ccccaaagac
2161 aaaccaagtg gagacacagc agctgtattt gaagaaggtg gtgatgtgga cgatttattg
2221 gacatgatat aggtcgtgga tgtatgggga atctaagaga gctgccatcg ctgtgatgct
2281 gggagttcta acaaaacaag ttggatgcgg ccattcaagg ggagccaaat tctcaagaaa
2341 ttcccagcag gttacctgga ggcggatcat ctaattctct gtggaatgaa tacacacata
2401 tatattacaa gggataattt agaccccata caagtttata aagagtcatt gttatttttct
2461 ggttggtgta ttatttttttc tgtggtctta ctgatctttg tatattacat acatgctttg
2521 aagtttctgg aaagtagatc ttttcttgac ctagtatatc agtgacagtt gcagcccttg
2581 tgatgtgatt agtgtctcat gtggaaccat ggcatggtta ttgatgagtt tcttaaccct
2641 ttccagagtc ctcctttgcc tgatcctcca acagctgtca cagcttgtgt tgagcaagca
2701 gtagcatttg cttcctccca acaagcagct gggttaggaa aaccatgggt aaggacggac
2761 tcacttctct ttttagttga ggccttctag ttaccacatt actctgcctc tgtatatagg
2821 tggttttctt taagtggggt gggaagggga gcacaatttc ccttcatact ccttttaagc
2881 agtgagttat ggtggtggtc tcatgaagaa aagacctttt ggcccaatct ctgccatatc
2941 agtgaacctt tagaaactca aaaactgaga aatttactac agtagttaga attatatcac
3001 ttcactgttc tctacttgca agcctcaaag agagaaagtt tcgttatatt gg
//
```

APPENDIX 10

☐ **1:** J03798. Human autoantigen...[gi:338264] Related Sequences, OMIM, Protein, PubMed, Taxonomy, LinkOut

```
LOCUS       HUMSNRNPD    1633 bp    mRNA            PRI       03-AUG-1993
DEFINITION  Human autoantigen small nuclear ribonucleoprotein Sm-D mRNA,
            complete cds.
ACCESSION   J03798
VERSION     J03798.1  GI:338264
KEYWORDS    autoantigen; ribonucleoprotein.
SOURCE      Human B-lymphocyte, cDNA to mRNA, clone D45-2.
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 1633)
  AUTHORS   Rokeach,L.A., Haselby,J.A. and Hoch,S.O.
  TITLE     Molecular cloning of a cDNA encoding the human Sm-D autoantigen
  JOURNAL   Proc. Natl. Acad. Sci. U.S.A. 85, 4832-4836 (1988)
  MEDLINE   88263041
COMMENT     Draft entry and computer-readable sequence [1] kindly submitted by
            L.Rokeach 20-JUL-1988.
            The Sm-D protein coded by cDNA D45-2, being a snRNP, is evidently
            involved in the mRNA splicing of highter eukaryotes; in the
            autoimmune disease systemic lupus erythematosus, antinuclear
            antibodies are developed with Sm specificity.
FEATURES             Location/Qualifiers
     source          1..1633
                     /organism="Homo sapiens"
                     /db_xref="taxon:9606"
     RBS             143..149
                     /note="ribosome binding site"
     CDS             151..510
                     /note="small nuclear riboprotein Sm-D"
                     /codon_start=1
                     /protein_id="AAA36620.1"
                     /db_xref="GI:338265"
                     /translation="MKLVRFLMKLSHETVTIELKNGTQVHGTITGVDVSMNTHLKAVK
                     MTLKNREPVQLETLSIRGNNIRYFILPDSLPLDTLLVDVEPKVKSKKREAVAGRGRGR
                     GRGRGRGRGRGRGGPRR"
BASE COUNT      474 a    278 c    373 g    508 t
ORIGIN      1 bp upstream of EcoRI site.
        1 gaattccccc ccccccccca gtgctccgcg cgctcttgac gtccggagcc cctggagtag
       61 gcgcttccgg ccattcatac tgcagtcggt cagtgttcgg ttgaaggatt ctgtgtgctg
      121 tcggacccag agggtgacgg cgccgctagg atgaagctcg tgagattttt gatgaaattg
      181 agtcatgaaa ctgtaaccat tgaattgaag aacggaacac aggtccatgg aacaatcaca
      241 ggtgtggatg tcagcatgaa tacacatctt aaagctgtga aaatgaccct gaagaacaga
      301 gaacctgtac agctggaaac gctgagtatt cgaggaaata acattcggta ttttattcta
      361 ccagacagtt tacctctgga tacactactt gtggatgttg aacctaaggt gaaatctaag
      421 aaaagggaag ctgttgcagg aagaggcaga ggaagaggaa gaggaagagg acgtggccgt
      481 ggcagaggaa gaggggtcc taggcgataa tgtctctcaa gatttcaaag tcatatgaga
      541 tttgggatat tttttgtaca ggttgtgttt gtttatgtca gtttttaata aacataaatg
      601 tgggacagag ctgtctattt agtatatcaa agttttagta gtttcctcca cattcacgaa
```

```
     661 attaccacag tgagagctaa gcatttctac tgggcagttt cattttagt tgatcaggtt
     721 ttaagttttt gaactaaaat ttttcttttt ctttttatga tgaataaggt taaaataaaa
     781 gccttagaca aattaaattt ggcagagttt aattgagcaa aggacaattc acaaatcagg
     841 tagcccctga accataatag gctcagaggc ttcagcccag ctgcatagtt gaagatttat
     901 ggacagaagg aaagtgatgt atggaaaatg gaagtgagat acagcaacag ccggattagt
     961 tacagttcag cgtttgcctt atttgaatat ggtttgaaca gttcgctgtc tttggttggc
    1021 tgaaacttag tgattgccac aagagtaggg taccgtctgt ttacacgtcc agttaggcta
    1081 cagttctatg tactgagaaa cctttaagct gaacttgaga tatgtaaaga gactttaggc
    1141 taaacttaac aatatatata ggaatatatc ccttctactt cacatgcact gaatatgcat
    1201 tttattgctt tactcttcat tctgtggcac ctacccacag gggaagtaag aagtttgttt
    1261 tggtatttcg gaaactaaag tccttatggg atggggtcta gaattgattc tcctttcctg
    1321 agttttactc cacggagtct taggtacctg gtaaaaagtt gtcttctaaa ttaagggtca
    1381 ttgctttgtt gtctagctgc taatgtctta cttttgtttc ttttgctttt taatcagttc
    1441 ttaataggat atagttttat gttttccaag ttataacttg gagttaatgg tcactagatt
    1501 atcagttatg agcagtgtta aaatctccta ttaatgtgta atgtacctgt cagtgcctcc
    1561 tttattaagg ggttctttga gaataaaaga gaaaagacct actttatttg acagcaaaaa
    1621 aaaaaaggaa ttc
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```

APPENDIX 11

☐ 1: M24499. Human cytochrome ...[gi:522194] Related Sequences, Protein, PubMed, Taxonomy, LinkOut

```
LOCUS       HUMP450DB1   1195 bp    mRNA            PRI       04-AUG-1994
DEFINITION  Human cytochrome P450db1 mRNA, 3' end, clone HLD8.2.
ACCESSION   M24499
VERSION     M24499.1  GI:522194
KEYWORDS    cytochrome P450db1.
SOURCE      Homo sapiens (individual_isolate Hepatitis patient) liver cDNA to
            mRNA.
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 1195)
  AUTHORS   Manns,M.P., Johnson,E.F., Griffin,K.J., Tan,E.M. and Sullivan,K.F.
  TITLE     Major antigen of liver kidney microsomal autoantibodies in
            idiopathic autoimmune hepatitis is cytochrome P450db1
  JOURNAL   J. Clin. Invest. 83, 1066-1072 (1989)
  MEDLINE   89155788
COMMENT     On Aug 5, 1994 this sequence version replaced gi:341176.
FEATURES             Location/Qualifiers
     source          1..1195
                     /organism="Homo sapiens"
                     /isolate="Hepatitis patient"
                     /db_xref="taxon:9606"
                     /tissue_type="liver"
     CDS             <1..1122
                     /codon_start=1
                     /product="cytochrome P450db1"
                     /protein_id="AAA36403.1"
                     /db_xref="GI:522195"
                     /translation="GPAWREQRRFSVSTLRNLGLGKKSLEQWVTEEAACLCAAFANHS
                     GRPFRPNGLLDKAVSNVIASLTCGRRFEYDDPRFLRLLDLAQEGLKEESGFLREVLNA
                     VPVLLHIPALAGKVLRFQKAFLTQLDELLTEHRMTWDPAQPPRDLTEAFLAEMEKAKG
                     NPESSFNDENLRIVVADLFSAGMVTTSTTLAWGLLLMILHPDVQRRVQQEIDDVIGQV
                     RRPEMGDQAHMPYTTAVIHEVQRFGDIVPLGVTHMTSRDIEVQGFRIPKGTTLITNLS
                     SVLKDEAVWEKPFRFHPEHFLDAQGHFVKPEAFLPFSAGRRACLGEPLARMELFLFFT
                     SLLQHFSFSVPTGQPRPSHHGVFAFLVTPSPYELCAVPR"
     polyA_signal    1174..1179
     polyA_site      1195
BASE COUNT      215 a    397 c    342 g    241 t
ORIGIN
        1 gggcccgcgt ggcgcgagca gaggcgcttc tccgtctcca ccttgcgcaa cttgggcctg
       61 ggcaagaagt cgttggagca gtgggtgacc gaggaggccg cctgcctttg tgccgccttc
      121 gccaaccact ccggacgccc ctttcgcccc aacggtctct tggacaaagc cgtgagcaac
      181 gtgatcgcct ccctcacctg cgggcgccgc ttcgagtacg acgaccctcg cttcctcagg
      241 ctgctggacc tagctcagga gggactgaag gaggagtcgg gctttctgcg cgaggtgctg
      301 aatgctgtcc ccgtcctcct gcatatccca gcgctggctg gcaaggtcct acgcttccaa
      361 aaggctttcc tgacccagct ggatgagctg ctaactgagc acaggatgac ctgggaccca
      421 gcccagcccc cccgagacct gactgaggcc ttcctggcag agatggagaa ggccaagggg
      481 aaccctgaga gcagcttcaa tgatgagaac ctgcgcatag tggtggctga cctgttctct
```

```
     541 gccgggatgg tgaccacctc gaccacgctg gcctggggcc tcctgctcat gatcctacat
     601 ccggatgtgc agcgccgtgt ccaacaggag atcgacgacg tgatagggca ggtgcggcga
     661 ccagagatgg gtgaccaggc tcacatgccc tacaccactg ccgtgattca tgaggtgcag
     721 cgctttgggg acatcgtccc cctgggtgtg acccatatga catcccgtga catcgaagta
     781 cagggcttcc gcatccctaa gggaacgaca ctcatcacca acctgtcatc ggtgctgaag
     841 gatgaggccg tctgggagaa gcccttccgc ttccaccccg aacacttcct ggatgcccag
     901 ggccactttg tgaagccgga ggccttcctg cctttctcag caggccgccg tgcatgcctc
     961 ggggagcccc tggcccgcat ggagctcttc ctcttcttca cctccctgct gcagcacttc
    1021 agcttctcgg tgcccactgg acagccccgg cccagccacc atggtgtctt tgctttcctg
    1081 gtgaccccat ccccctatga gctttgtgct gtgccccgct agaatggggt acctagtccc
    1141 cagcctgctc cctagccaga ggctctaatg tacaataaag caatgtggta gttcc
//
```

APPENDIX 12

☐ **1:** M28639. Human autoimmune ...[gi:291864] Related Sequences, PubMed, Taxonomy

```
LOCUS       HUMATDRAGA    572 bp    mRNA            PRI       12-JUN-1993
DEFINITION  Human autoimmune thyroid disease-related antigen mRNA.
ACCESSION   M28639
VERSION     M28639.1  GI:291864
KEYWORDS    autoimmune thyroid disease-related antigen.
SOURCE      Homo sapiens Graves' thyroid cDNA to mRNA.
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 572)
  AUTHORS   Hirayu,H., Seto,P., Magnusson,R.P., Filetti,S. and Rapoport,B.
  TITLE     Molecular cloning and partial characterization of a new autoimmune
            thyroid disease-related antigen
  JOURNAL   J. Clin. Endocrinol. Metab. 64, 578-584 (1984)
  MEDLINE   87138159
FEATURES             Location/Qualifiers
     source          1..572
                     /organism="Homo sapiens"
                     /db_xref="taxon:9606"
                     /tissue_type="thyroid"
                     /dev_stage="Graves'"
     mRNA            <1..>572
                     /note="antigen is not thyroglobulin, the microsomal
                     antigen, or the TSH-binding receptor"
BASE COUNT      162 a    155 c    125 g    130 t
ORIGIN
        1 aagtcccagc ctcccgctgc cgtccagtgt gtgtactgta cacatccaca ctcactctca
       61 ctcagggttc ccggaccggc tgtcctgcct gcggaactga ggtaaacaag ctcaggtact
      121 gacactagga gggtctacct tacataaggt acaggtagaa gcttgattga ttgctaggcc
      181 caggccaccc agaccctcca atcctaacag gtatttaggc ttgaggttca ctccctcctc
      241 agctgcacac gcagccaggt ataacactcg ccctcagtca caacggggag ggggcaccgg
      301 ttacatctac atcacattat ttataaaata agaattacat ttcatataac atggccagaa
      361 ggagctctag tcccccagga aagctgccgg gacagcattt gagcctcttc tttgcacagg
      421 cataacttaa ctatacagct aattgggggg ggccccccct gaaaaataat ccccccaaat
      481 aagcagcatg tcatgtcctt agaatatgtt acactagaaa ggtagaaaaa ttcaggctaa
      541 ggaggcaatt tagagtcaag ttatcacgaa tt
//
```

APPENDIX 13

☐ 1: M64695. Synthetic human a...[gi:208505] Related Sequences, Protein, PubMed, Taxonomy

```
LOCUS       SYNHUMACRS               645 bp    DNA     linear   SYN 27-APR-1993
DEFINITION  Synthetic human acetylcholine receptor alpha subunit gene, complete
            cds.
ACCESSION   M64695
VERSION     M64695.1  GI:208505
KEYWORDS    acetylcholine receptor subunit-alpha.
SOURCE      Synthetic DNA.
  ORGANISM  synthetic construct
            artificial sequences.
REFERENCE   1  (bases 1 to 645)
  AUTHORS   Talib,S., Leiby,K.R., Wright,K. and Okarma,T.B.
  TITLE     Cloning and expression in Escherichia coli of a synthetic gene
            encoding the extracellular domain of the human muscle acetylcholine
            receptor alpha-subunit
  JOURNAL   Gene 98 (2), 289-293 (1991)
  MEDLINE   91200680
   PUBMED   2016069
FEATURES             Location/Qualifiers
     source          1..645
                     /organism="synthetic construct"
                     /db_xref="taxon:32630"
     gene            6..641
                     /gene="acetylcholine receptor alpha subunit"
     CDS             6..641
                     /gene="acetylcholine receptor alpha subunit"
                     /codon_start=1
                     /transl_table=11
                     /product="acetylcholine receptor alpha subunit"
                     /protein_id="AAA73020.1"
                     /db_xref="GI:208506"
                     /translation="MSEHETRLVAKLFKDYSSVVRPVEDHRQVVEVTVGLQLIQLINV
                     DEVNQIVTTNVRLKQQWLDYNLKWNPDDYGGVKKIHIPSEKIWRPDLVLYNNADGDFA
                     IVKFTKVLLQYTGHITWTPPAIFKSYCEIIVTHFPFDEQNCSMKLGTWTYDGSVVAIN
                     PESDQPDLSNFMESGEWVIKESRGWKHSVTYSCCPDTPYLDITYHFVIQRL"
BASE COUNT      172 a    177 c    158 g    138 t
ORIGIN
        1 aattcatgtc cgaacatgag acccgtctgg tggcaaagct atttaaagac tacagcagcg
       61 tggtgcggcc agtggaagac caccgccagg tcgtggaggt caccgtgggc ctgcagctga
      121 tacagctcat caatgtggat gaagttaatc agatcgtgac aaccaatgtg cgtctgaaac
      181 agcaatggct ggattacaac ctaaaatgga atccagatga ctatggcggt gtgaaaaaaa
      241 ttcacattcc ttcagaaaag atctggcgcc cagaccttgt tctctataac aatgcagatg
      301 gtgactttgc tattgtcaag ttcaccaaag tgctcctgca gtacactggc cacatcacgt
      361 ggacacctcc agccatcttt aaaagctact gtgagatcat cgtcacccac tttccctttg
      421 atgaacagaa ctgcagcatg aagctgggca cctggaccta cgacggctct gtcgtggcca
      481 tcaacccgga aagcgaccag ccagacctga gcaacttcat ggagagcggg gagtgggtga
      541 tcaaggagtc ccggggctgg aagcactccg tgacctattc ctgctgcccc gacacccccc
      601 acctggacat cacctaccac ttcgtcatcc agcgcctgta atgaa
```

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Revised: October 24, 2001.

Jun 12 2002 10:51:26

APPENDIX 14

1: J Immunol Methods 1995 Jul 17;184(1):81-9 Related Articles, Books, LinkOut

Isolation of human blood dendritic cells by discontinuous Nycodenz gradient centrifugation.

McLellan AD, Starling GC, Hart DN.

Haematology/Immunology Research Laboratory, Christchurch Hospital, New Zealand.

The most potent antigen presenting cell present in peripheral blood, lymphoid and non-lymphoid tissue is the dendritic cell (DC). The study of human DC has been restricted by their low frequency in the tissues and the lack of a truly DC specific surface marker to assist in identification and isolation. Standard techniques for the isolation of blood DC generally employ a period of in vitro culture followed by flotation on dense albumin gradients, or more recently, discontinuous gradients of metrizamide. Dense albumin gradients are time consuming to prepare, giving low and variable yields of DC. Metrizamide is more convenient, although exposure of monocytes to metrizamide can decrease the expression of CD14 and alter the accessory cell properties of antigen presenting cells. Here we demonstrate that Nycodenz gradient centrifugation of 16 h cultured, T lymphocyte depleted, peripheral blood mononuclear cells (PBMC) reliably yields a population of low density cells that is highly enriched for DC. Most B and residual T lymphocytes are depleted and NK cell numbers are reduced two-fold from the interface cell population. The high density pellet fraction exhibits very little allostimulatory activity, indicating that few DC pass into the pellet. The low density fraction contains a significant population (20 +/- 5 (SD)%, n = 8) of cells which fail to stain for the lineage markers CD3, CD11b, CD14, CD16, CD19 and CD57. Nycodenz exhibits low toxicity, does not alter the allostimulatory activity of antigen presenting cells, and is therefore ideal for the isolation of cultured DC.

PMID: 7622872 [PubMed - indexed for MEDLINE]

sparc-sun-solaris2.8 Oct 4 2001 12:24:52

AACN Clinical Issues
Volume 7, Number 2, pp. 212–220

APPENDIX 15

Current Red Blood Cell Transfusion Practices

Lawrence Tim Goodnough, MD

The appropriate use of blood transfusions remains variable among health-care institutions and patient populations. Transfusion practices are discussed in this article in relation to medical practice guidelines and utilization review. Specific transfusion practices in the settings of intensive care, orthopedic surgery, and open heart surgery are reviewed. A new, promising approach to improving transfusion outcomes is the use of transfusion algorithms. Transfusion algorithms may prove especially useful if they incorporate point-of-care testing that is both physiologic and patient-specific for transfusion decisions. Transfusion algorithms are discussed and data presented for cardiac surgical adults (KEYWORDS: transfusion practices, medical practice guidelines, transfusion algorithms).

Medical practice guidelines are being promoted as a means to improve medical care.[1] Guidelines for transfusion practices can contribute to improved care only if they change physician transfusion behavior. However, guidelines are unlikely to change behavior unless there are incentives for physicians to do so.[2] In the absence of incentives, there is widespread skepticism about the value of guidelines or recommendations from consensus conferences. One promising approach is to guide the decision-making process by coupling the use of algorithms for the transfusion of blood and blood components with readily-available clinical information obtained from point-of-care testing, rather than laboratory-based assays. An overview of current transfusion practices and their relation to guidelines and the process of utilization review is presented.

Guidelines and Utilization Review: Are They Effective?

A recent review concluded that transfusion audits can improve transfusion practices if they are performed in a timely manner and are combined with education of the individual ordering physician.[3] Plasma and platelet products are particularly amenable to this approach. Two studies using concurrent education or consultation reduced plasma usage by 46% and 77%, respectively.[4,5] In another study, using a retrospective audit, inappropriate plasma use was reduced from 53% to 22% of units transfused.[6] Similarly, use of platelet transfusions were reduced by 56% and 14% in two studies that used consultation[7] and au-

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dit,[8] respectively. Another study followed all requests for transfusions with a nonrequested consultation. The authors report a reduction in transfusion of platelets by 44%, and plasma and cryoprecipitate by 57%, but a reduction in erythrocytes of only 19% during a 4-year period.[9] Other studies cast doubt on whether utilization review is really an effective process. Hoeltge et al.[10] used a combination of indicators to evaluate transfusions on medical and surgical services, and concluded that only 4% of transfusions were unjustified. Renner and colleagues[11] found the percentage of unjustified transfusions to be 1.4% before and 0% after an educational intervention. The percentages of transfusions that failed initial screening were also extraordinarily low: only 0.5% and 3.2%, respectively, before and after the intervention. At our own institution in 1994, in which 23,002 erythrocyte units were transfused, 48 transfusion events underwent peer review by the transfusion committee and only 2 cases (or a rate of 1 in 10,000) were thought to be unjustified.

These extraordinarily low rates of "inappropriate" transfusions may be a consequence of several factors. First, erythrocyte transfusion audits in circumstances of hemorrhage are difficult, if not impossible, to evaluate. These settings would include the emergency room/trauma unit, the operating rooms, and the intensive care units. For this reason, our institutional process of utilization review excludes transfusions administered intraoperatively. Yet, studies of transfusion practices in orthopaedic surgery indicate that at least 25% of erythrocyte transfusions in this setting can be, in retrospect, identified to be inappropriate.[12] Second, the clinical indicators that define "appropriate" transfusion practice may be too generous. In the study that concluded that 96% of transfusions were "appropriate," a posttransfusion hemoglobin level of 11 gm% was used as a threshold to distinguish "appropriate" from "inappropriate."[10] Third, the medical chart audit has substantial limitations. Clearly documented information as to why the transfusion was administered is commonly unobtainable. We found that in orthopaedic surgical patients, only 68% of postoperative transfusion events on the day of surgery had chart documentation of blood loss and/or change in vital signs,[13] laboratory hematocrit levels of 33.5% ± 0.9% before transfusion, and 31.3 ± 0.5% after transfusion. In addition, the rationale for transfusion was recorded in only 16% of day-of-surgery transfusions and in only 27% of transfusions administered on postoperative days. At our own institution, 9 of 48 cases reviewed by the transfusion committee throughout 12 months were thought to have inadequate documentation for the reason for transfusion.

With these limitations, one alternative approach to retrospective chart audit for utilization review is the prospective use of transfusion algorithms, in which the decision process is coupled with information that serves as clinical indicators for transfusion. These algorithms could potentially have significant impact in the intensive care units and in the surgical setting. Therefore, current transfusion practices in these settings are reviewed.

☐ Transfusion Practices in the Intensive Care Setting

A recent study of transfusion practices in the intensive care unit was performed in 4,875 consecutive patients admitted to 6 Canadian tertiary-level intensive care units.[14] Overall, 28% of the patients received erythrocyte transfusions. However, the number of transfusions per patient-day ranged from 0.82 to 1.08 among institutions, with a mean of 0.95 units/patient per day. The authors found the institutional effect on this variability to remain significant even after adjusting for age and APACHE II score. They found that the most frequent reasons for administering erythrocyte were acute bleeding (35%) and the augmentation of oxygen delivery (25%). This latter observation may explain why, in a recent study,[5] altering physicians' transfusion trigger based on hemoglobin levels did not affect the transfusion outcomes in an intensive care setting. In this study, transfusion guidelines based on hemoglobin levels (as low as 7.0 gm% for asymptomatic patients) could successfully alter blood ordering practices, but they were not able to reduce overall blood use.[15] The mean hemoglobin level at transfusion was decreased from 8.5 gm% to 8.1 gm%, but the proportion of patients transfused and the number of units trans-

fused (per patient, or patient-day) did not change. As noted in another study,[14] patients are more likely to receive blood transfusions that are triggered because of hemorrhage, ischemia, or issues related to oxygen delivery.

In a recent study of critically ill patients with sepsis, a set of physiologic goals to optimize oxygen delivery (>600 mL/min/m^2), was evaluated in a series of interventions, including fluid boluses, administration of blood products, and the use of inotropes.[16] Whereas the intervention group had the same complications, number of days on ventilator and in intensive care, and hospital charges compared with a standard therapy group (oxygen delivery goal of 450–550 mL/min/m^2), the patients who did achieve oxygen delivery in excess of 600 mL/min/m^2 (in both groups) had significantly less mortality (14%) compared with patients who could not maintain this high level of oxygen delivery (mortality of 56%). This study illustrates the paradigm that interventions, including blood products, that are keyed to patient-specific physiologic clinical indicators can result in better clinical outcomes.[17]

☐Transfusion Practices in Orthopaedic Surgery

Estimates of total hip replacements (135,000), total knee replacements (110,000), and hemiarthroplasties of the hip (77,000) suggest that 5% of all erythrocyte units transfused in the United States in 1987 went to patients undergoing joint replacement (DRG 209) surgery.[18,19] An analysis of blood transfusions among 6,472 patients in 151 hospitals who underwent joint replacement surgery in 1986 (a year in which only 1.5% of erythrocyte transfused were autologous) was performed.[17] In this study, it was found that 46% and 68% of patients who underwent knee and hip replacement, respectively, received blood transfusions. The authors observed an influence of gender on transfusion outcomes. Male and female patients undergoing primary hip replacement were transfused in 60% and 73%, respectively, of cases; in primary knee replacement, males and females were transfused in 36% and 50% of cases.

The influence of gender on transfusion outcomes was noticed previously. Friedman and co-workers[20] found that women and men were discharged from surgical services with similar hematocrit levels, despite lower admission hematocrit levels in women; this was a result of increased blood transfusions to female surgical patients relative to their male counterparts. This phenomenon was studied further in a single institution study of 525 elective orthopaedic surgical patients,[12] in which female patients were found to be overtransfused when compared with male patients. Clinical indicators that ranged from "generous" to "strict" for transfusion inappropriateness were analyzed (Table 1). The excessive transfusion rates were seen especially in the 70% of patients who had predonated autologous blood: 50% of these autologous blood donors were transfused in excess of needs, according to an "intermediate" clinical indicator, compared with 24% of patients who had not predonated autologous blood.

The discharge hematocrit levels for orthopaedic patients at our institution during this era ranged from 30.8% to 33.7%,[21] further underscoring the likelihood that perisurgical anemia was being treated too aggressively with blood transfusion therapy. A multicenter study at six hospitals also found similarly high discharge hemoglobin levels, ranging from 10.7 gm% to 11.0 gm% for patients undergoing joint replacement surgery.[22] Therefore, whereas autologous blood donation practices substantially reduced the likelihood of receiving allogeneic blood during the latter 1980s from 41% to as low as 18% of orthopaedic patients,[23] on the basis of the above studies, a considerable percentage of elective surgical patients received blood transfusions in excess of their needs.[24,25] Some of these patients could be identified to be at risk for transfusion because of preexisting anemia. In one study,[26] 34 (21%) of 162 consecutively evaluated orthopaedic patients had preoperative hematocrit levels of less than 39%. Therefore, patient-specific factors can be identified as predeterminants of the need for transfusion.

In addition to patient-specific factors, however, institutional-dependent factors may be responsible for transfusion outcomes. In one report of transfusion outcomes in patients who did not predonate autologous blood, the erythrocyte units transfused for initial total hip replacement surgery was 1.7

TABLE 1 ■ Blood Transfusions in Complex* Orthopaedic Surgery

Clinical Indicator	Percent of Patients Transfused in Excess of Blood Needs	p Value
"Generous" (O.K. if erythrocyte losses ≥ 10% of baseline)		
Men	13	<0.001
Women	25	
"Intermediate" (O.K. if erythrocyte losses ≥ 20% of baseline)		
Men	33	<0.001
Women	49	
"Strict" (O.K. if erythrocyte losses ≥ 30% of baseline)		
Men	52	<0.001
Women	66	

*Hip revision, bilateral knee, and bilateral hip procedures.

Adapted, with permission, from Goodnough LT, et al. Transfusion 1992;32:64.

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± 2.9[21]; yet another institution from the same time period reported 2.3 ± 1.5 units for the same procedure.[22] More recently, 382 Medicare patients at 5 hospitals in Massachusetts who underwent orthopaedic surgery were audited for transfusion outcomes.[2] The authors found substantial variability in the availability of autologous blood, either as predeposit (range 58–88%) or as intraoperative salvage (range 0–75%). They concluded that blood transfusions were avoidable in 37% of autologous transfusion episodes and in 10% of allogeneic transfusion episodes. They also found that the mean transfusion hematocrit levels were significantly higher for autologous units compared with allogeneic units: 28.4% versus 25.7%, respectively.

In summary, there is much evidence to suggest that transfusion practices in orthopaedic surgery remain in evolution. Despite diminished use of allogeneic blood in this setting, there is need for a more standardized approach to the application of various technologies used in autologous blood procurement, as well as a more standardized approach to transfusion practices.

Transfusion Practices in Cardiac Surgery

The evolution of cardiac surgery has been accompanied by blood conservation interventions that combined blood salvage techniques[28] along with acceptance of postoperative normovolemic anemia. This resulted in a single institution report of allogeneic erythrocyte transfusions in as few as 10% of patients undergoing elective coronary artery bypass graft (CABG) surgery.[29] However, in a later study from the same institution, researchers reported allogeneic blood transfusions in 40% of patients, largely as a result of changing patient demographics such as increasing age and redoes.[30] Considerable variation in transfusion practice among institutions was identified. A multicenter audit of 18 institutions demonstrated a wide range in allogeneic erythrocyte transfusion requirements for patients undergoing simple, first-time CABG surgery.[31] This variability was confirmed in two subsequent studies.[32,33] Follow-up studies of transfusion outcomes in cardiac surgical patients indicate that a substantial number of blood components in patients are transfused inappropriately.[34]

Issues of blood safety in transfusion medicine renewed interest in blood conservation and alternatives to blood transfusion.[35] Practice guidelines were summarized by the National Institutes of Health consensus conferences on perioperative transfusion of erythrocytes.[36] Yet, as these guidelines have suggested hemoglobin thresholds as low as 70 g/L for transfusion in surgical patients, concern was raised over whether the pendulum has swung too far.[37] Hematocrit levels of 18% were described to be as well tolerated by patients as levels of 27% during cardiac bypass.[38] Yet, patients were reportedly at risk for perioperative ischemic injury

in the setting of postoperative hemodilution to hematocrit levels that range from 21% to 24%, with a delay in myocardial metabolic recovery.[39] Hemoglobin levels were suggested as clinical indicators for transfusion in patients undergoing a coronary bypass[40]:

1. Hemoglobin of 60 g/L for well-compensated chronically anemic patients; healthy (ASA Class I and some Class II) patients undergoing intentional hemodilution; and patients undergoing hypothermic cardiopulmonary bypass.
2. Hemoglobin of 80 g/L for most postoperative bypass patients except those with left ventricular hypertrophy, incomplete coronary revascularization, low cardiac output, poorly controlled tachycardia, or sustained fever.
3. Hemoglobin of 100 g/L for patients unlikely to increase cardiac output, patients with symptomatic cerebrovascular disease, and elderly (age > 65) patients.

Others emphasize that it is unlikely that any hemoglobin level can be universally applicable.[41] More physiologic indicators of the adequacy of oxygen delivery were proposed.[42] The routine placement of a thermodilution pulmonary artery catheter in patients undergoing CABG surgery enables regular assessment of mixed venous oxygen saturation (SVO_2), along with hemodynamic variables such as cardiac index, heart rate, and blood pressure. Mixed venous oxygen saturation is an indicator of the relative balance between the total body oxygen supply and demand. As a sensitive but nonspecific indicator, SVO_2 represents a weighted balance from all perfused vascular beds. When arterial oxygen saturation is adequate (> 0.90%), the SVO_2 inversely reflects the oxygen supply–demand balance. Mixed venous oxygen saturation provides continuous quantification of global oxygen extraction, in which a mixed venous oxygen tension greater than 40 mmHg (SVO_2 approximately 75%) is believed to indicate adequate tissue oxygenation in most clinical states, and mixed venous oxygen tension less than 20 torr (SVO_2 approximately 30%) suggests inadequate tissue oxygenation.[42] The understanding that hemoglobin is a poor clinical indicator of erythrocyte mass and tissue oxygen delivery[43] focused attention on the importance of more physiologic indicators for blood transfusion in this setting.

Therefore, in addition to clinical indicators for erythrocyte transfusion (tachycardia, hypotension, oliguria, etc), physiologic indicators that indicate clinically significant impairment in the balance of oxygen supply and demand are also important. When the hemoglobin level falls below "acceptable" values, these physiologic indicators indicate a potential benefit from transfusion. However, the precise "acceptable" hemoglobin concentration range is unknown. The National Institutes of Health consensus conference concluded that most patients with hemoglobin levels greater than 100 g/L do not require blood, whereas most patients with hemoglobin levels less than 70 g/L benefit from blood.[36] However, silent perioperative ischemia was identified as a significant clinical problem in noncardiac[44] as well as cardiac[45] surgical patients, emphasizing that the heart is an organ at risk with regard to tissue oxygenation. In a recent case report, researchers illustrated that in a surgical patient whose hematocrit level was 27%, silent myocardial ischemia was corrected with the transfusion of two erythrocyte units.[46] Nelson et al.[47] reported that in high-risk vascular surgery patients, a mean hematocrit level of less than 28% on the first postoperative day was associated with myocardial ischemia in 10 of 13 patients and a morbid cardiac event in 6 of these patients. Therefore, hemoglobin levels from 70 g/L to 100 g/L, a range in which physiologic indicators may identify patients who can benefit (or not benefit) from blood, need to be the most closely scrutinized.

In previous studies, researchers found that although the erythrocyte needs of these patients are substantial, a combination of conservative transfusion practice and blood conservation that could provide the equivalent of four blood units might avoid allogeneic blood exposure in as many as 75% of patients undergoing CABG surgery.[48] Because 80% of erythrocyte transfusions are received on the postoperative day and 90% by the end of postoperative day 1, the effectiveness of interventions focused on these 2 days would be effective. Patients were found to be transfused in excess of their needs if decisions to transfuse are made on a clinical indicator, such as estimated blood loss, that

is poorly quantifiable.[48] Transfusion algorithms, therefore, may be especially useful if they incorporate point-of-care information that is both physiologic and patient-specific for transfusion decisions.

Transfusion Algorithms

Although the discharge hematocrit level is useful in a retrospective understanding of transfusion outcomes,[21] transfusion guidelines using concurrent clinical indicators are necessary if physician transfusion behavior is to be altered. We conducted studies to evaluate the impact of point-of-care testing,[49,50] in which intraoperative assays (on-site evaluation of whole blood prothrombin time, activated partial thromboplastin time, and platelet count, with results available within 4 minutes) were linked with a transfusion algorithm (Figure 1) for plasma and platelet transfusions in cardiac surgical patients.

This algorithm approach reduced blood component transfusions for the algorithm-treated patients group, compared with patients treated with standard practice using laboratory-based testing. Sixty-six patients with a diagnosis of microvascular bleeding were randomized to either standard therapy (n = 36), in which blood products were transfused at the discretion of the physician according to any laboratory-based test results requested; or to an algorithm group (n = 30), in which on-site platelet count, prothrombin time, and activated partial thromboplastin time results were available within 4 minutes. Platelet and plasma therapy were given according to an algorithm, according to on-site results.

The three decision pathways of this algorithm were based initially on platelet count measurements, followed by branch pathways determined by prothrombin time and activated partial thromboplastin time results. Both intraoperative and initial postoperative chest tube drainage were less in the algorithm group, indicating that hemostatic therapy was more successful in treating microvascular bleeding in the algorithm group. Point-of-care testing and patient-specific (i.e., targeted to each patient's laboratory test results) blood component therapy may have better distinguished hemostatic versus surgical bleeding for the surgical team at the close of the procedure, as suggested by the fact that only one patient in the algorithm group required later surgical reexploration, compared with five pa-

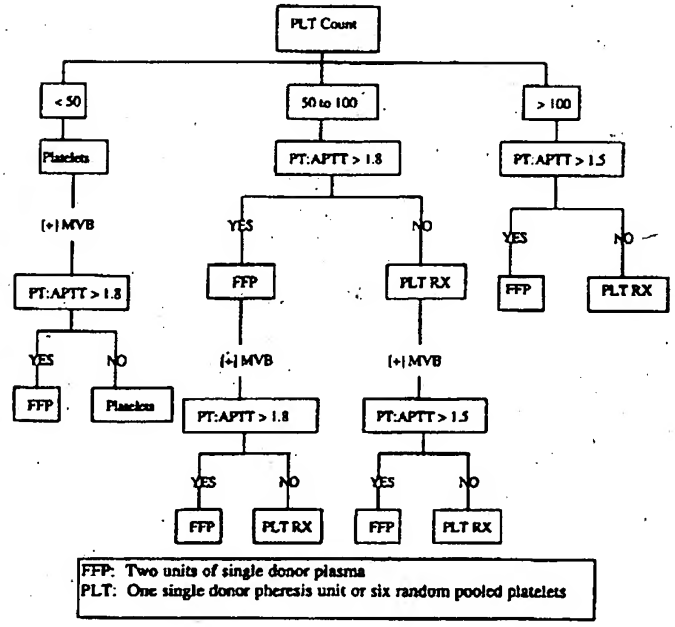

Figure 1. An algorithm approach for hemostatic therapy in cardiac patients determined to have microvascular bleeding after heparin neutralization. Platelets = platelet transfusion (6 units of random-donor or apheresis unit equivalent); PLT RX = platelet therapy (platelet transfusion and/or desmopressin acetate therapy at physician's discretion); FFP = plasma therapy (2 units of fresh-frozen plasma); [+] MVB = continued microvascular bleeding; PT:APTT = whole blood prothrombin time and activated partial thromboplastin time control values (values/mean values from a normal reference population); PLT count = platelet count (× 10³/mm³). Reprinted with permission from Despotis GJ, Grishaber JE, Goodnough LT. The effect of an inoperative treatment algorithm or physician transfusion behavior in cardiac surgery. Transfusion 1994; 34:290–296.

tients in the standard group. Eight (25%) of 36 standard therapy patients received different blood component therapy from what would have been designated by our algorithm.[50] The more effective therapy in the algorithm group was reflected in the lower erythrocyte transfusion needs in the algorithm group compared with the standard therapy group (5.9 ± 3.8 versus 9.8 ± 8.4 units, respectively). The improved patient care along with reduced blood transfusions resulted in substantial economic savings. This approach was described as a "powerful engine of change."[51]

Decisions to transfuse the surgical patient should acknowledge that patients are heterogeneous for risks related to anemia. One prospective approach to risk stratification for cardiac surgical patients has been published recently,[52] in which clinical variables determine whether a patient is considered as a "standard" or "increased" risk for cardiac surgery. Although complications related to anemia represent only one aspect of morbidity/mortality in this setting, the physiologic changes known to accompany acute anemia,[53] and the potential for myocardial tissue injury,[54] suggest that risk stratification for erythrocyte transfusion decisions would be prudent. A recent analysis of more than 2,000 cardiac bypass patients led to a model that calculated a transfusion risk score, which was then validated prospectively in more than 400 additional patients.[53] Now that cardiac surgery patients can be prospectively stratified not only for surgical risk but also for transfusion likelihood, the role of algorithms may be especially productive in the standardization of blood transfusion and blood conservation practices.

An algorithm that incorporates the balance between oxygen delivery and oxygen consumption as reflected by changes in SVO_2 within a range of hematocrit level, may be effective as a clinical indicator for erythrocyte therapy. Such an approach is illustrated in Figure 2.[54] With the recognition that transfusion support is dependent on both rate of blood loss and hemoglobin level, the decision to transfuse each unit of erythrocyte could be based on hemoglobin level, the quantity (rate) of blood lost, and hemodynamic parameters. Each patient would achieve adequate pulmonary capillary wedge pressures filling pressures with crystalloid/colloid therapy before entering a transfusion algorithm. Controlled clinical trials comparing this approach to current, unmonitored transfusion practices are needed.

If transfusion outcomes can be predicted from patient-related factors, then blood transfusion and blood conservation algorithms can be used to minimize the variability of transfusion outcomes related to institutional (procedural) and physician (transfusion practices)

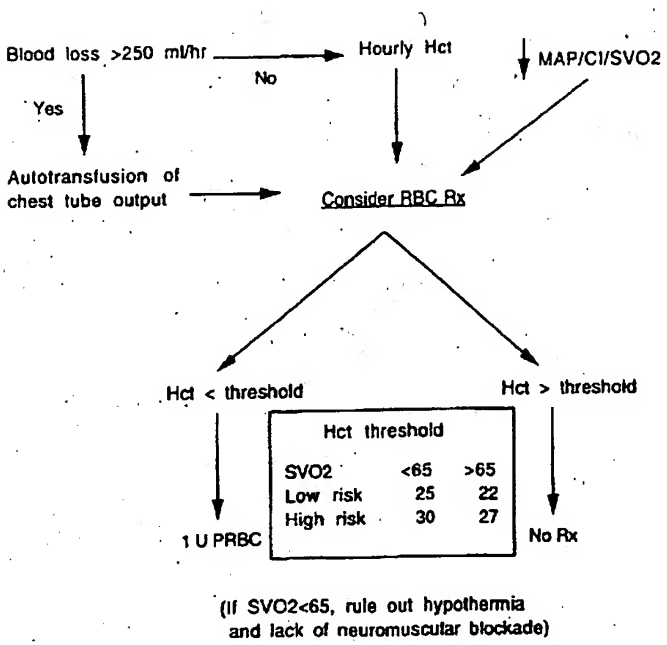

Figure 2. An algorithm approach for erythrocyte transfusion in cardiac surgical patients postoperatively. After establishing that the patient's volume status is adequate, decisions to transfuse would be based on hemoglobin/hematocrit level, rate of blood loss, and hemodynamic parameters. Thresholds for transfusion would differ for patients determined to be at "low" risk and "high" risk for perisurgical complications. Mixed venous oxygen percent saturation (SVO_2) could serve as a physiologic indicator of the balance between oxygen supply and demand for transfusion decision-making. Reprinted with permission from Goodnough LT, Despotis GJ, Hogue CW, Ferguson TB. On the need for improved transfusion indicators in cardiac surgery. Ann Thoracic Surg 1996;61:27–32.

factors. Such algorithms could take into account patient heterogeneity by using the Higgins et al.[52] and McGovern et al.[53] stratification of "standard" and "increased" risk patients. Blood transfusions and blood conservation strategies could be administered according to algorithms incorporated into the daily practice of coronary revascularization.[51] Such an approach can then enable physicians to analyze transfusion outcomes, and the relation of these outcomes, to transfusion triggers, autologous blood procurement, pharmacologic interventions, and even emerging blood substitutes. The knowledge that we learn from the standardization of these practices, and the comparison of transfusion outcomes between different institutions or different therapeutic approaches, would form an important database that could serve as a reference for the continuous improvement of care in the surgical setting.[55]

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APPENDIX 16

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Prolonged production of NADPH oxidase-corrected granulocytes after gene therapy of chronic granulomatous disease

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ABSTRACT **Little is known about the potential for engraftment of autologous hematopoietic stem cells in human adults not subjected to myeloablative conditioning regimens. Five adult patients with the p47^{phox} deficiency form of chronic granulomatous disease received intravenous infusions of autologous CD34$^+$ peripheral blood stem cells (PBSCs) that had been transduced *ex vivo* with a recombinant retrovirus encoding normal p47^{phox}. Although marrow conditioning was not given, functionally corrected granulocytes were detectable in peripheral blood of all five patients. Peak correction occurred 3–6 weeks after infusion and ranged from 0.004 to 0.05% of total peripheral blood granulocytes. Corrected cells were detectable for as long as 6 months after infusion in some individuals. Thus, prolonged engraftment of autologous PBSCs and continued expression of the transduced gene can occur in adults without conditioning. This trial also piloted the use of animal protein-free medium and a blood-bank-compatible closed system of gas-permeable plastic containers for culture and transduction of the PBSCs. These features enhance the safety of PBSCs directed gene therapy.**

Chronic granulomatous disease (CGD) is a rare inherited disorder of phagocytes associated with recurrent life-threatening infections (1, 2). CGD is caused by a defect in the phagocyte NADPH oxidase (phox) that normally generates superoxide. When normal phagocytes engulf opsonized pathogens, the oxidase becomes activated by translocation of three cytoplasmic proteins (p47^{phox}, p67^{phox}, and rac-2) to the cell membrane where they bind to flavocytochrome b_{558} (a heteromeric transmembrane protein composed of two peptides, gp91^{phox} and p22^{phox}) (3, 4). The genetic basis of CGD is heterogeneous (1, 5). The most common form (about two-thirds of the cases) is X chromosome-linked, resulting from mutations in the gp91^{phox} gene. The next most common form (about one-third of the cases) is autosomal recessive resulting from mutations in the p47^{phox} gene on chromosome 7 (2, 4). The remaining 5% of cases are due to mutations in the genes encoding p22^{phox} (chromosome 16) or p67^{phox} (chromosome 1).

Bone marrow transplantation can cure CGD (6, 7), indicating that the stem cells giving rise to granulocytes and monocytes are an appropriate target for gene therapy. Bone marrow transplantation in CGD has been associated with unacceptably high rates of morbidity, mortality, and graft failure, except in the case of HLA-matched sibling donors (6, 7). Specific gene therapy of autologous peripheral blood stem cells (PBSCs) would avoid these problems. The feasibility of genetic correction of CGD with retrovirus vectors has been demonstrated *ex vivo* by transduction of human CD34$^+$ PBSCs from patients with each of the four forms of CGD (8–10). Furthermore, genetic correction of the gp91^{phox} and p47^{phox} deficiency forms of CGD has been demonstrated *in vivo* after stem-cell gene therapy of gene knockout CGD mice and is associated with an increased resistance to infection (11, 12).

In the CGD mouse gene therapy studies, total body radiation was used as a conditioning regimen to enhance engraftment of gene corrected stem cells. Although partial marrow ablation has been thought to be required to optimize engraftment of infused hematopoietic stem cells even in the autologous setting, a number of animal studies using syngeneic cells have suggested that infusion of large numbers of stem cells can partially overcome this barrier (13). In this clinical trial of gene therapy, we examine the potential for engraftment of transduced-gene-corrected autologous CD34$^+$ stem cells in adult patients with the p47^{phox} deficiency form of CGD (p47^{phox} CGD) without marrow conditioning.

MATERIALS AND METHODS

Patients and Consent Documents. Patients 1 to 5 have p47^{phox} CGD as demonstrated by history of recurrent infections, by phagocytic cells that lack both oxidase activity and p47^{phox} protein, and by p47^{phox} gene mutation analysis (14, 15). Patients 1 to 5 are Caucasian and are, respectively, female, male, female, male, and female, and years of age at study entry were 37, 21, 18, 27, and 27. A gene-therapy phase I protocol with associated informed consent document was reviewed and approved by the National Institute of Allergy and Infectious Disease human investigation review board (Protocol 95-I-0134), by the National Institutes of Health Biosafety Committee (Approval document RD-94-XI-05), by the National Institutes of Health Recombinant DNA Advisory Committee (Protocol 9503–104), and by the U.S. Food and Drug Administration (BB IND 6100).

Protocol Clinical Procedures. Beginning on study day 1, patients were given six daily subcutaneous injections with granulocyte colony-stimulating factor (Amgen) at 10 μg/kg to mobilize CD34$^+$ PBSCs from the marrow (16). On both study days 5 and 6, a 10- to 15-liter apheresis stem cell collection was performed by using the CS3000 Plus blood cell separator

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Abbreviations: PBSC, peripheral blood stem cell; CGD, chronic granulomatous disease; phox, phagocyte NADPH oxidase; PMA, phorbol myristate acetate; NBT, nitroblue tetrazolium dye; DHR, dihydrorhodamine 123.

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23.6×10^6 cells ($n = 10$) was recovered, at a median purity of 80% $CD34^+$ cells resulting in a median yield of 38%.

After the 3 days of culture and transduction, each patient received two autologous products (Table 1, preparations A and B) without any symptoms or changes in vital signs. The total number of cells infused ranged from 0.1 to 4.7×10^6 cells per kg (Table 1). Transduced PBSCs averaged 91 ± 2.7% cell viability, had a colony plating efficiency of 104 ± 38.4 colonies per 1,000 cells plated, and passed safety testing for sterility, endotoxin, and replication competent retrovirus. SDS/PAGE and immunoblotting demonstrated a strong positive signal for the presence of recombinant $p47^{phox}$ protein in all of the transduced CGD PBSC cultures (Fig. 1, lanes B and C). Shown in Table 1 are studies performed on each product to assess the percent of cells expressing CD34 antigen on culture day 3, correction of oxidase activity by chemiluminescence and DHR assays on culture day 17, the percent of myeloid-colony-forming progenitors plated on day 3 giving rise to oxidase-positive colonies, and the vector copy number in the transduced PBSCs. The data are consistent with preservation of a primitive phenotype and a high rate of gene transfer and functional correction *ex vivo*. Though not shown, nontransduced PBSC cultures from the 10 apheresis products from the five patients demonstrated less than 1% of normal chemiluminescence and DHR assay and gave rise to no NBT-positive myeloid colonies. When 17-day-cultured PBSCs from the CGD patients were compared with cultured PBSCs from normal individuals, similar numbers of granulocytes are present by morphological examination in transduced and nontransduced cultures of CGD PBSCs and in the cultures of normal PBSCs, but highly fluorescent oxidase positive cells were detected in the flow cytometry DHR assay only in transduced cell cultures from patients as reported in Table 1 and from normal individuals. Though not shown, the mean fluorescence per corrected patient granulocyte in the transduced cultures was similar to that seen with granulocytes derived from cultured normal PBSCs, suggesting full restoration of oxidase activity in gene-corrected granulocytes *ex vivo*.

Presence of NADPH Oxidase-Positive Neutrophils in the Peripheral Blood After Intravenous Administration of *Ex Vivo* Transduced Autologous $CD34^+$ PBSCs. The flow cytometry DHR assay was used to measure the appearance of NADPH oxidase-positive granulocytes in the peripheral blood after gene therapy. The dot plot shown in Fig. 2*A* demonstrates that the events generated from analysis of PMA-stimulated normal peripheral blood granulocytes cluster in a tight band at the right side of the graph, consistent with robust oxidase activation. As shown in Fig. 2*B*, analysis of PMA-stimulated blood granulocytes from patient 1 before gene therapy resulted in all but a single event to the left of the "positive threshold" line, characteristic of absent oxidase function. At day 24 after gene therapy, PMA-stimulated peripheral blood granulocytes from patient 1 generated almost 80 events appearing to the right of the "positive threshold" line in a tight cluster (Fig. 2*C*) with mean fluorescence intensity (x axis) similar to that of granulocytes from the normal control (compare Fig. 2 *A* and *C*). Although the number of corrected cells is small, the data indicate that these gene corrected granulocytes from patient 1 have acquired oxidase activity similar to normal cells. Qualitatively similar results were obtained for the other four patients. To confirm the results of the DHR assay by direct visualization of individual neutrophils, an NBT stain of PMA-stimulated peripheral blood neutrophils was performed. In the example shown in Fig. 3 with blood from patient 1 at day 26 after gene therapy, 1 in 2,000 granulocytes was oxidase-positive, consistent with the count determined by DHR assay as reported in Fig. 4.

Each patient was followed over time for detection of corrected granulocytes in peripheral blood (Fig. 4). In each subject no oxidase-corrected granulocytes were detected for at least 2 weeks

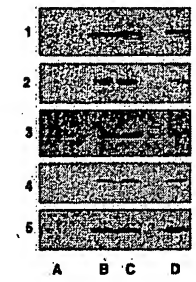

FIG. 1. Correction of $p47^{phox}$ protein deficiency *ex vivo*. These are SDS/PAGE immunoblots demonstrating detection of $p47^{phox}$ protein in transduced or control $CD34^+$ PBSCs at culture day 17. The results for studies of patients 1 to 5 are shown from the top to bottom, as indicated. Shown in lanes A are the analyses of nontransduced cultured $CD34^+$ PBSCs from each patient and as expected no signal is detected. Shown in lanes B and C are the analyses of cultured and MFGS-$p47^{phox}$-transduced $CD34^+$ PBSCs derived from the first and second apheresis products from each patient. Shown in lanes D as a positive control in each case is an analysis of nontransduced normal control $CD34^+$ PBSCs cultured in parallel with the patient cells.

Table 1. Evaluation of autologous PBSC after *ex vivo* transduction and culture

Patient*	Prep	Cells infused†	% $CD34^+$‡	% of normal chemiluminescence§	DHR assay,§ % granulocytes corrected	% NBT-positive colonies¶	Vector copy number‖
1	A	60	85	25	30	9	0.05
	B	200 (4.7)	97	26	21	6	0.11
2	A	12	84	26	90	29	0.19
	B	45 (0.9)	94	23	44	28	0.08
3	A	215	61	65	75	14	0.13
	B	66 (4.3)	85	64	75	18	0.18
4	A	77	92	27	34	9	0.16
	B	129 (2.5)	81	36	63	11	0.11
5	A	2	63	32	27	19	0.13
	B	2 (0.1)	79	39	59	14	0.13

*Each patient received by vein two preparations (Prep, A and B) of the transduced and cultured PBSCs derived from the first and second apheresis procedures, respectively.

†At culture day 3 for each preparation, the number of cells shown ($\times 10^{-6}$) were infused intravenously. Shown in the parentheses is the total number of cells ($\times 10^{-6}$) (A plus B) infused per kg of body weight.

‡Measured by flow cytometry analysis at the end of culture day 3.

§Assays were performed on day 17 of culture.

¶Cells were plated at culture day 3 and assayed 14 days later.

‖Measured by Southern blot of genomic DNA from the transduced and cultured PBSCs, probed with a MFGS-vector-specific 5′ long terminal repeat sequence and a cell line with known vector copy number of 1 as a reference.

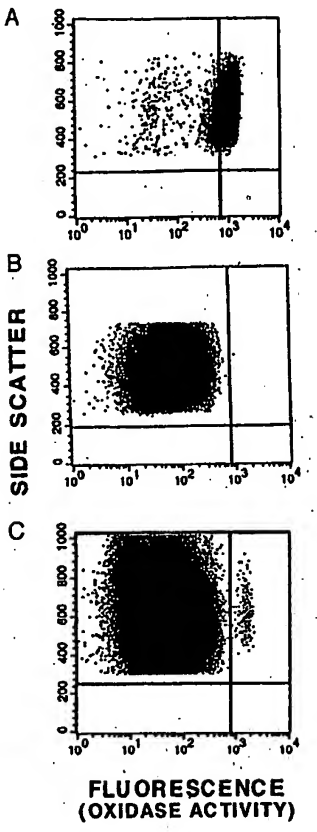

FIG. 2. Correction of neutrophil oxidase activity *in vivo*. These are dot plots of the flow cytometry DHR assays of oxidant production by PMA-stimulated peripheral blood granulocytes. Shown are analyses of granulocytes. (*A*) Normal volunteer. (*B*) Patient 1 before gene therapy. (*C*) Patient 1 at 24 days after gene therapy. Each event (dot) represents the analysis of parameters derived from a single cell. The data shown have been gated to include only events with the forward × side scatter characteristics of granulocytes. Data are plotted to evaluate fluorescence (*x* axis) as a measure of oxidase activity and side scatter (*y* axis) as a means of distinguishing individual granulocytes (events). The "positive threshold" vertical line is set so that 95% of stimulated normal granulocytes are to the right of that line in the region defined as oxidase positive.

after transplantation of the autologous transduced PBSCs. After that time, an increasing number of oxidase-positive granulocytes could be detected in the peripheral blood. The peak response occurred between day 25 (patient 3) and day 53 (patient 2) with a mean of 35 days. The maximum percent of oxidase-positive granulocytes at the peak ranged from 0.004% (patient 2) to 0.051% (patient 1) with a mean of 0.019% (about 1 in 5,000 cells). The range of duration of detection of oxidase-corrected granulocytes was 51 days (patient 5) to 172 days (patient 3) with a mean of 118 days. Of note is that the kinetics of appearance of oxidase-corrected cells did not rise to a single peak followed by a smooth decay over time but instead appeared to rise and fall several times over the duration of detection of positive signal. For example, patient 3 demonstrates five (possibly six) maxima at days 25, possibly 32, 39, 53, 102, and 172.

It is of note that the greatest number of transduced PBSCs were administered to patients 1, 3, and 4 in that rank order and that these same three patients showed the longest duration of detection of corrected granulocytes. Furthermore, patients 1 and 3 had the highest peak number of such corrected granulocytes. Patients 2 and 5, in that rank order, had far fewer transduced PBSC transfused and this correlated with patients 2 and 5, in that order,

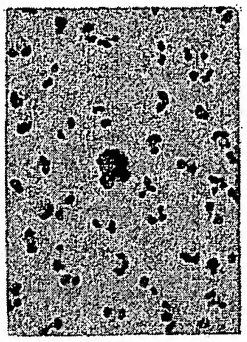

FIG. 3. Correction of neutrophil oxidase activity *in vivo*. This photomicrograph shows NBT-stained PMA-stimulated neutrophils from peripheral blood of patient 1 at day 26 after gene therapy. The cytospin preparation is counterstained red-orange with safranin to visualize segmented neutrophil nuclei. Shown in the center is a single NBT-positive neutrophil that is partly obscured by the dense blue-black precipitate of formazan, a product of NBT reduction by superoxide. This amount of precipitate is evidence of vigorous production of superoxide by this gene-therapy-corrected neutrophil. A visual count of neutrophils on this slide showed that about 1 in 2,000 cells were NBT-positive.

having the shortest duration and patient 2 having the lowest peak number of corrected granulocytes. Patients 1 to 5 have now been followed after gene therapy for 25, 23, 22, 21, and 20 months (as of August 1997), respectively, but no oxidase-positive cells have been seen in any peripheral blood DHR assay after the last data points shown in Fig. 4. PCR detection of the transduced $p47^{phox}$ cDNA was performed on genomic DNA isolated from peripheral blood leukocytes as shown in Table 2 and confirms that a transient low level of gene marking of peripheral blood leukocytes occurred.

Results of Safety Studies and Long-Term Clinical Follow Up. PCR assay of amphotropic envelope sequence failed to detect evidence of replication-competent retrovirus in peripheral blood leukocytes at 1, 3, 6, and 9 months after gene therapy. Similarly, serum samples were negative for anti-$p47^{phox}$ antibodies at 1 and 3 months after gene therapy. All five patients are currently stable without infection. Except for patient 1, the other four patients have had no deep tissue infections during the follow-up period, and hematologic, renal, and liver function tests are normal. Patient 1 had severe *Burkholderia cepacia* pneumonia 3 weeks after gene therapy, from which she recovered. During her pneumonia oxidase-positive neutrophils were detected in an empyema by flow cytometry DHR assay and NBT stain, demonstrating that these gene therapy corrected cells were capable of migrating to an inflammatory focus. It is possible that host responses to this infection affected the peak level of gene corrected granulocytes seen in this individual.

DISCUSSION

Our data demonstrate the appearance of gene-corrected oxidase-positive granulocytes in the peripheral blood of each of five patients with $p47^{phox}$ CGD after PBSC-targeted gene therapy with vector encoding $p47^{phox}$. In patient 1, we also demonstrated that the gene-corrected oxidase-positive neutrophils could migrate from the circulation to a site of infection. Moreover, the kinetics of appearance of these functionally corrected granulocytes share similar characteristics in all patients. In all patients, the first appearance of oxidase-positive granulocytes required at least 2 weeks, suggesting that engraftment, proliferation, and differentiation of the transduced PBSCs in the marrow was required. In all patients, there was an initial wave of oxidase-positive cells first peaking at 22–39 days followed by one or more waves (usually of much smaller magnitude) of oxidase-positive cells at intervals out to 6 months in some individuals. If this periodicity is real, it could be evidence of clonal succession where only a small subset

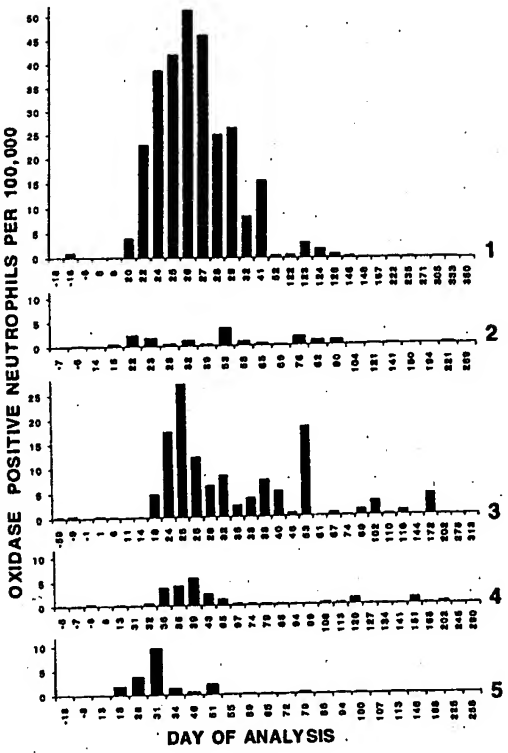

FIG. 4. Prolonged production of oxidase-corrected granulocytes *in vivo*. These bar graphs demonstrate over time the proportion of oxidase-positive neutrophils in the peripheral blood after gene therapy of the five CGD patients. For each data point, flow cytometry DHR assay was performed on peripheral blood leukocytes and the number of oxidase-positive neutrophils was determined as shown in Fig. 2. From top to bottom, the results from analyses of blood from patients 1 to 5, respectively, are shown. For all patients, the vertical axis (oxidase-positive neutrophils per 100,000 cells) is the same scale allowing direct visual comparison between patients. However, the horizonal axis is not proportional and the numbers beneath the data bars indicate the days of analyses relative to the first intravenous administration of transduced autologous $CD34^+$ PBSCs and differ for each patient.

of primitive progenitors in the marrow contribute to active hematopoiesis at any time (25).

Most studies of gene marking of autologous PBSCs have subjected patients to the myeloablative conditioning usually associated with cancer therapy (ref. 26; for review, see ref. 27). Less is known about engraftment potential of autologous progenitors without ablative marrow conditioning. With some disorders such as adenosine deaminase-deficient severe combined immune deficiency or Fanconi syndrome, gene-therapy-corrected lymphocytes or hematopoietic progenitors may have a selective growth advantage over uncorrected cells (28–31). In reports of the use of transduced hematopoietic blood stem cells to treat adenosine deaminase-deficient severe combined immune deficiency without myeloablation, there is evidence of prolonged engraftment (30, 31). The three newborn infants with adenosine deaminase-deficient severe combined immune deficiency treated with transduced autologous cord blood stem cells show evidence of rising levels of gene-marked lymphocytes, though marking of myeloid cells remains very low in a range similar to that seen in our present study (31). Because correction of adenosine deaminase deficiency should provide a selective growth advantage specifically to lymphocytes, this finding might have been expected. In addition, cord blood is a particularly rich source of stem cells and infants and very young children may be more receptive to engraftment of autologous cells. It remains to be seen whether

Table 2. PCR detection of gene marking

Pt 1		Pt 2		Pt 3		Pt 4		Pt 5	
Day*	PCR†	Day	PCR	Day	PCR	Day	PCR	Day	PCR
−6	−	−3	−	−8	−	−6	−	−15	−
28	+	24	+/−‡	3	−	29	+	−3	−
33	+	28	−	26	+	33	+	29	−
124	−	68	−	55	+	40	+	34	+
147	−	93	−	70	+	65	−	61	−
241	−	190	−	92	−	89	−	82	−
313	−	357	−	119	−	118	−	117	+
362	−	147	+	132	−	139	−		
		175	−	352	−	335	−		
		205	−						
		232	−						

Pt, patient.
*Number of days before or after gene therapy.
†Vector $p47^{phox}$ cDNA sequence detected (+) or not detected (−) in blood leukocytes. All + were <0.1% of cells marked.
‡The +/− indicates positive signal detected by Southern blotting of the PCR product but not by nested primer detection.

permanent high-level engraftment of autologous-gene-marked hematopoietic stem cells can be achieved in nonconditioned older children or adults, where gene transfer provides no selective growth advantage.

We have demonstrated in a congenic mouse model system that low-dose nonablative radiation conditioning can increase greatly the engraftment of congenic marrow stem cells in a radiation dose-dependent manner (32). This suggests the possibility that acceptable regimens of marrow conditioning may be developed for hematopoietic-stem-cell-targeted gene therapy. Such conditioning might increase the level of gene marking from that seen in our current study to levels that could provide prolonged clinical benefit.

The DHR assay of oxidase function provides strong evidence that low-level prolonged engraftment of gene-marked hematopoietic progenitors can occur in human adults without marrow ablation or conditioning. Though the PCR confirmed this, PCR was not sensitive enough to detect marking at later time points where the flow cytometry DHR assay continued to indicate lower levels of oxidase-positive neutrophils. The PCR data demonstrate that the eventual disappearance of gene-corrected oxidase-positive granulocytes by DHR assays was not associated with continued presence in the peripheral blood of substantial numbers of leukocytes marked with a nonfunctional transduced gene. Although we cannot exclude the possibility that silencing of transcription of the transduced oxidase gene is occurring, the data are more consistent with disappearance of transduced cells. This might happen if very early progenitors rather than permanently repopulating stem cells were targeted. Several published human clinical studies of hematopoietic-stem-cell-targeted gene transfer (26, 27, 30, 31) have demonstrated low-level engraftment of retrovirus-transduced gene in blood or marrow cells by using PCR. Although these studies indicate the presence of the transduced gene, assessment of gene function in the host, as in our study and others (33), provides an important additional insight regarding the clinical potential for gene therapy. How to target the most primitive stem cells and how to prevent transcription silencing *in vivo* remain important issues to resolve in future studies.

An additional goal of this clinical trial was to develop and pilot the use of materials and methods that increase the safety of *ex vivo* gene therapy targeting hematopoietic stem cells. Animal proteins, including fetal calf serum, are widely used as required supplements to most cell culture media. Animal proteins internalized by human cells during prolonged culture may not be removed by centrifugation washing and can stimulate an immune response (34). Because gene therapy is in an early developmental stage, it is likely that any patients participating in these initial

studies will be treated again in the future at a time when such treatments are more efficient. If it is at all possible to limit exposure to animal proteins, particularly fetal calf serum, in these early studies without compromising the scientific goals of the study, then such a safety feature should be incorporated into the protocol. A second important safety feature incorporated into this study was a closed system of gas-permeable flexible plastic containers for culture and transduction. The closed system reduces the contamination risk associated with pipetting cells and medium yet allows such handling to become a counter-top process. Biosafety cabinets are required at only a few steps and the system is compatible with techniques already used widely in most blood banks. We demonstrate that it is possible to incorporate these safety features without compromising PBSC viability or transduction efficiency.

The clinical potential of gene therapy is yet to be realized, and there has been considerable interest in defining both the scientific and clinical goals of human trials of gene transfer. In the case of CGD, where life-threatening infections may require many weeks or months of therapy and relapses are frequent, use of gene therapy to provide even short- to medium-term production of oxidase-positive autologous granulocytes may be clinically beneficial. This concept is supported by published studies of gene therapy in mouse models of both the X chromosome-linked ($gp91^{phox}$-deficiency) and $p47^{phox}$-deficiency forms of CGD that demonstrate that even transient partial correction of the oxidase defect is associated with some protection against infection challenge (11, 12). Furthermore, in human female carriers of the X chromosome-linked form of CGD, the X chromosome inactivation that occurs during embryogenesis results in phenotypic mosaicism at the cellular level in which both oxidase-positive and oxidase-negative granulocytes can be detected in the peripheral blood (21). Because this is a stochastic process, some female carriers can be found who have only 3% to 5% oxidase-positive neutrophils yet do not suffer from an increased incidence of infection. The knockout mouse studies and the clinical observations of X chromosome-linked CGD carriers suggest that even a short-term low level of gene correction in CGD could be clinically beneficial for treatment of severe prolonged infections. Until the tools are developed to achieve high-level permanent gene transfer to hematopoietic cells, our studies suggest that an achievable intermediate goal of development of gene therapy for CGD might be to augment neutrophil function in the treatment of severe infections.

Somatix Therapy Corporation, an industrial collaborator during the conduct of this study, is now a part of Cell Genesys. We thank Immunex for providing Pixykine for *ex vivo* culture of $CD34^+$ PBSCs. We are grateful for the important contributions of the National Institute of Allergy and Infectious Diseases 11 East day hospital staff and the National Institutes of Health transfusion medicine apheresis staff. We thank Dr. Stephen Chanock for doing the $p47^{phox}$ mutation analysis of our patients. We thank Dr. Philip Murphy for critical reading of the manuscript and Dr. Douglas Kuhns for preparing Fig. 3. Finally, we thank the participating patients and the physicians who served as physician-advocates for their patients during the informed consent process.

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APPENDIX 17

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of
Daniel B. Drachman

Group Art Unit: 1633

Serial No.: 09/205,096

Examiner: E. Sorbello

Filed: December 3, 1998

Attorney Docket No.: 01107.77737

For: TARGETING ANTIGEN-SPECIFIC CELLS FOR SPECIFIC IMMUNOTHERAPY OF AUTOIMMUNE DISEASE

DECLARATION UNDER 37 C.F.R. RULE 1.132

Honorable Assistant Commissioner
for Patents
Washington, DC 20231

Sir:

I, Daniel B. Drachman, hereby declare:

1. THAT, I am the inventor of the invention described and claimed in the application named above. I am a medical doctor. Presently, I am a full professor of Neurology and Neurosciences and the Director of the Neuromuscular Clinic and Research Laboratory at Johns Hopkins University School of Medicine.

2. THAT, we have demonstrated experimentally that antigen presenting cells ("APCs") transduced by vaccinia virus vectors ("VVV") engineered to express acetylcholine receptors ("AChR"), or AChR and Fas ligand ("FasL"), or AChR and FasL and truncated FADD, caused effective and specific killing of AChR-specific T-cells in culture.

3. THAT, the experimental system employed standard and well-known methods and materials which are also described in the captioned patent application.

Briefly, a first genetic element contained the extracellular domain of the α subunit of Torpedo AChR (amino acids 1-210) functionally connected to the mouse LAMP1 signal sequence and a transmembrane/cytoplasmic tail. A second genetic element

encoding FasL was optionally inserted into VVV. A third element encoding truncated FADD was optionally inserted into VVV. The elements, alone or in various combinations, were inserted into VVV by homologous recombination. The VVV employed was the wt WR strain from ATCC which was attenuated by psoralen UV irradiation. Radio-labeled T-cell lines specific to AChR and/or keyhole limpet hemocyanin ("KLH") were produced by methods known in the art (stimulation by AChR or KLH, respectively) from Lewis rats and from lpr/lpr mutant rats (which do not express Fas and thus are not succeptable to FasL). Splenocytes from naïve mice or rats were used as APCs and were transduced with engineered or wt VVVs. The APC were tested to determine their effect on T-cells by co-incubation with the T-cells. Various controls, such as added purified acetylcholine receptor, were employed. As shown in Figure 3B (Exhibit A), mouse splenocytes transduced with the three genetic elements had a powerful destructive effect on the target cells (lymphoma cells). The effect was not seen when the genetic elements were used singly. Moreover, the effect was not seen when the target cells did not express Fas (lpr/lpr mice) as shown in Figure 6 (Exhibit A).

4. THAT, the data demonstrate that T-cells were targeted by the AChR extracellular domain and the killing was specifically mediated by FasL. The killing was very effective, because, after exposure to the relevant engineered APCs, no stimulation by added purified AChR receptor of T-cells specific for AChR was observed. The results indicate that the extracellular domain of the α subunit of AChR was sufficient to target virtually the entire population of AChR stimulated T-cells.

5. THAT, in a separate set of experiments, we have demonstrated that APCs transduced with VVV engineered to express influenza hemagglutinin ("HA") and FasL effectively and specifically killed HA-specific T-cells in culture. The materials and methods were similar to those described in the captioned patent application and paralleled the experimental approach described in paragraph 3, *supra*. VVV were engineered to express combinations of the HA gene, FasL gene, and/or truncated FADD gene. HA-specific T cells were used. Ovalbumin ("OVA")-specific T-cells were used as control cells. As shown in Figure 4 (Exhibit B), HA-specific T-cells were specifically and strongly growth inhibited by the triple gene construct containing HA, FasL, and truncated

FADD. T-cells of different specificity (OVA) were not inhibited by the same construct. When apoptosis was measured rather than mere growth inhibition, it was clear that the triple gene construct induced apoptosis far more effectively than anti-Fas antibody. See Figure 5 (Exhibit B). Thus, the triple gene therapy inhibits growth of specific T-cells and induces apoptosis of the specific T-cells.

6. THAT the data demonstrate that the VVV-transferred HA gene stimulated and targeted HA-specific T- cells, that FasL was effectively expressed from the VVV vectors, that effective HA-specific T-cells killing was specifically mediated by FasL. The killing was very effective and only HA-specific T-cells were targeted.

7. THAT the observations in paragraphs 3-6 demonstrate that the methods of the captioned application lead to effective presentation of antigens by APCs, and effective targeting and destruction of specific T-cells. Two different antigens were successfully tested according to the method of the invention.

8. THAT, all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 4/24/01

Daniel B. Drachman

EXHIBIT A

Legends:

Figure 3B Functional effect of FasL:

Balb/C mouse splenocytes were transduced by overnight infection with various attenuated VVV preparations @ 20 pfu/cell. A20 target cells were pre-labeled with [^{3}H]-TdR. The transduced splenocytes were incubated overnight with A20 target cells at different Effector:Target (E:T) ratios. Triplicate wells, each containing 2.5 x 10^4 A20 cells/well were used. APCs transduced with the "triple gene" VVV induced marked loss of radioactivity. APCs transduced with control VVV expressing TrFADD or Sig-AChR-LAMP1, or infected with wt VV - but without FasL - did not.

Abbreviations:

wt - APCs with wild type vaccinia virus.
TrFADD - APCs transduced with VVV expressing truncated FADD.
TAChR - APCs transduced with VVV expressing the Sig-AChR-LAMP1 gene construct.

Figure 6. Essential role of FasL in elimination of AChR-specific T cells by "3-gene" transduced APCs.

AChR-specific T cell lines were prepared from lymph node cells of C57B1/6 mice or lpr/lpr mutant mice (lacking Fas), that were immunized with AChR. Spleen cells from naive C57B1/6 mice were used as APCs. They were transduced with Sig-AChR-LAMP1 VVV (TAChR); or the AChR "3-gene" VVV (TAChR-FasL-TrFADD); or infected with attenuated control vaccinia (wt). AChR-specific T cells (5 x 10^4) from C57B1/6 mice, or from lpr/lpr mice, were incubated with 2.5 x 10^5 APCs in triplicate microwells for 5 days, and [^{3}H]-TdR was added for the final 8 h of the culture. The AChR-specific T cells from lpr mice proliferated in response to the "3 gene" VVV transduced APCs. The AChR-specific T cells from C57B1/6 mice were inhibited.

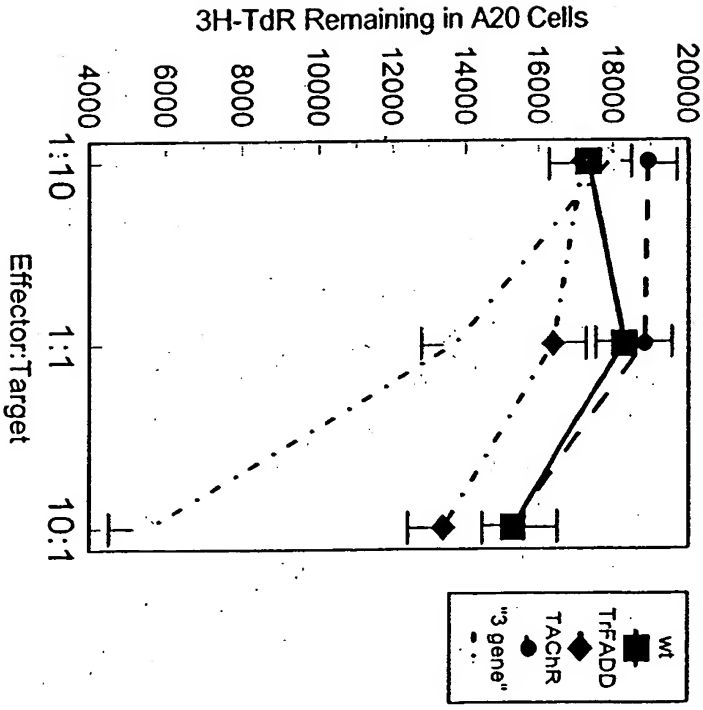
3H-TdR Remaining in A20 Cells
4000
6000
8000
10000
12000
14000
16000
18000
20000
1:10
1:1
10:1
Effector:Target
wt
TrFADD
TAChR
"3 gene"

FIGURE 3B

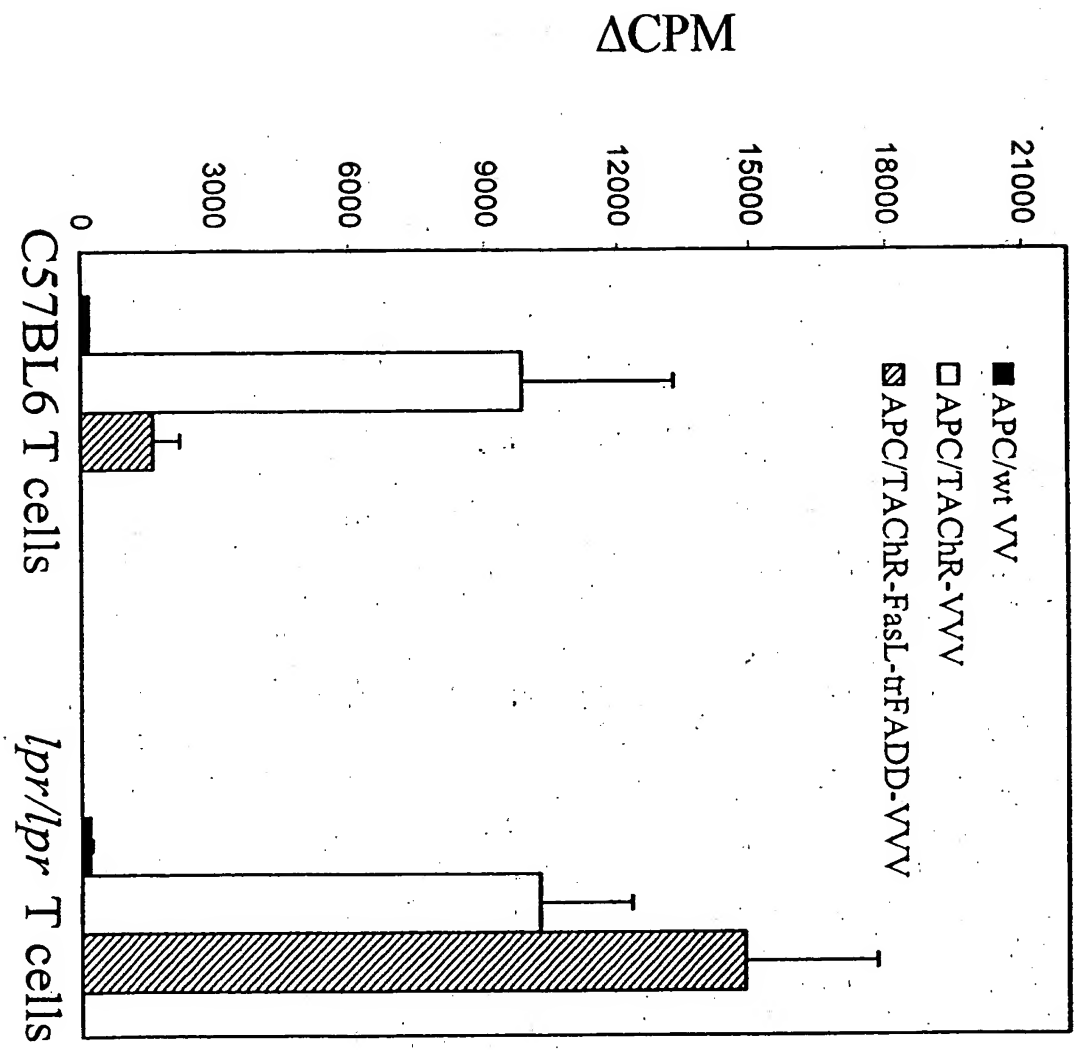
ΔCPM
21000
18000
15000
12000
9000
6000
3000
0
APC/wt VV
APC/TAChR-VVV
APC/TAChR-FasL-trFADD-VVV
C57BL6 T cells
lpr/lpr T cells

FIGURE 6

EXHIBIT B

Figure Legends:

Figure 4 Antigen specificity of effect by vvv-transduced APCs.

Lymph node cells from HA-specific TCR transgenic mice, and from OVA-specific TCR transgenic mice were pre-stimulated in separate bulk cultures with HA peptide (10 μg/ml) or OVA (40 μg/ml) respectively for 48 hours. HA-specific or OVA-specific T cells were then co-cultured with Balb/C splenocytes that had been infected with either attenuated "3-gene" vvv or with control wt vv for 5 days (2×10^4 T cells; 2×10^5 APCs, in triplicate cultures), and pulsed for the last 18 h with [^{3}H]-TdR. Results are expressed as Δcpm ± SEM. Note marked inhibition of HA-specific T cells, with negligible effect on the OVA-specific T cells.

Figure 5. Antigen targeting of vvv transduced APCs enhances FasL effect.

Spleen cells from HA-specific TCR transgenic mice were used as target T cells. They were pre-stimulated for 48 h with HA peptide (10 μg/ml), and then co-cultured overnight with Balb/C APCs infected with various attenuated vvv, as indicated (5×10^4 target cells and 5×10^5 APCs, in triplicate microcentrifuge tubes). The supernatant was collected, and fragmentation of DNA indicative of apoptosis was determined by an ELISA method that detects histone-bound mono- and oligonucleotides (see text). The background was measured from supernatants of vvv-transduced APCs alone. The results, given as the "enrichment factor", are considered positive if they are significantly greater than 1. Note that co-culture with "triple-gene" transduced APCs induced a marked increase of DNA fragmentation; co-culture with APCs transduced with FasL and TrFADD did not.

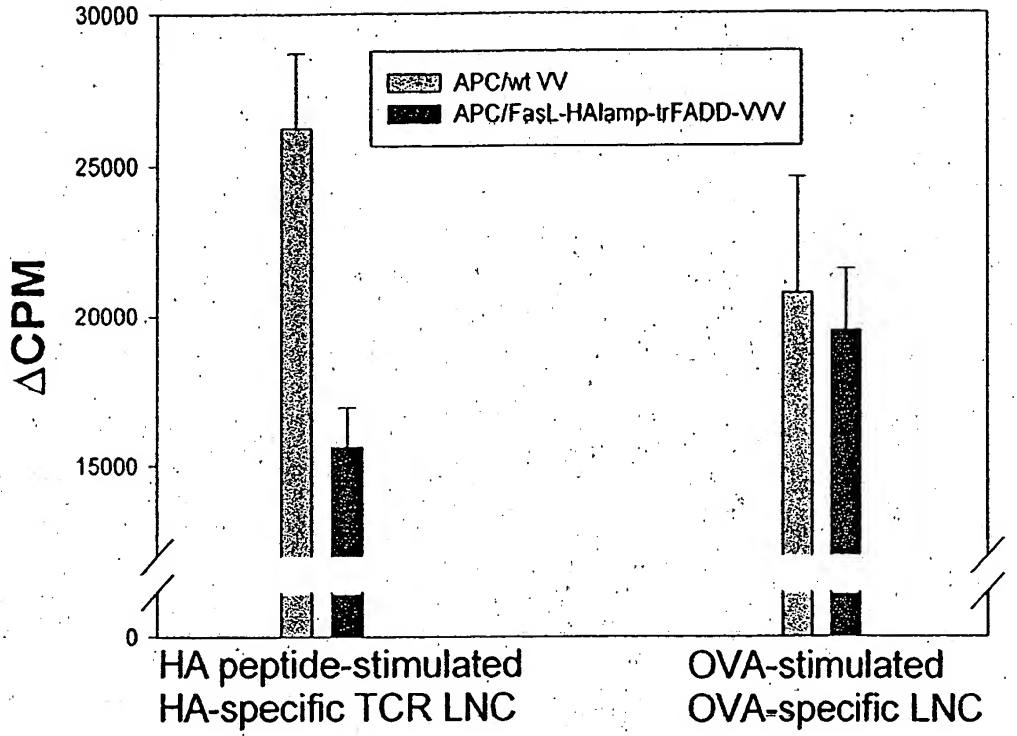
30000
25000
20000
15000
0
ΔCPM
APC/wt VV
APC/FasL-HAlamp-trFADD-VVV
HA peptide-stimulated
HA-specific TCR LNC
OVA-stimulated
OVA-specific LNC

FIGURE 4

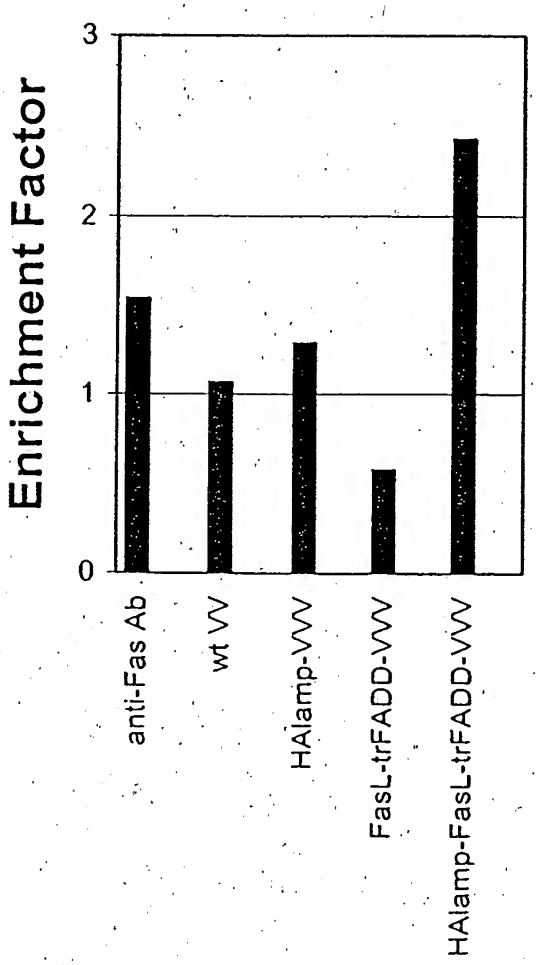
Enrichment Factor
3
2
1
0
anti-Fas Ab
wt VV
HAlamp-VVV
FasL-trFADD-VVV
HAlamp-FasL-trFADD-VVV

FIGURE 5

APPENDIX 18

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)
	) Group Art Unit: 1633
Daniel B. Drachman	)
	) Examiner: E. Sorbello
Serial No. 09/205,096	)
	)
Filing Date: December 3, 1998	) Docket No. 01107.77737

For: **TARGETING ANTIGEN-SPECIFIC CELLS FOR SPECIFIC IMMUNOTHERAPY OF AUTOIMMUNE DISEASE**

DECLARATION UNDER RULE 132

I, Daniel B. Drachman, hereby declare:

1. I am the sole inventor of the application referenced above.

2. I have conducted experiments that demonstrate that antigen presenting cells (APCs) transduced by vaccinia virus vectors (VVV) containing genes that encode the influenza hemagglutinin (HA) antigen, Fas ligand (FasL), and truncated FADD cause effective killing of HA-specific T cells in a transgenic mouse.

3. The transgenic mouse used in the experiments has T cells that express an HA-specific T cell receptor. The HA-specific T cell receptor is expressed in approximately 50% of the T cell population of the transgenic mouse.

4. Antigen presenting cells (APCs) specific for HA-specific T cells were prepared by isolating APCs from BALB/c mice and infecting them with an attenuated vaccinia virus vector (VVV). The VVV was genetically engineered to contain three genes encoding: HA (functionally connected to mouse LAMP1), Fas ligand, and truncated FADD. Control APCs were prepared by infecting APCs with an attenuated VVV encoding two genes: Fas ligand and truncated FADD gene. The control APCs thus did not contain the gene encoding HA.

5. An HA transgenic mouse as described in paragraph 3 was injected intraperitoneally

(IP) with $5.0x10^7$ APCs specific for HA-specific T cells as described in paragraph 4. A second HA transgenic mouse was injected IP with $5.0x10^7$ of the control APCs described in paragraph 4. Two HA transgenic mice served as untreated controls and did not receive an injection of APCs.

6. HA-specific T cell killing was measured in each of the HA transgenic mice by flow cytometry. Peripheral blood lymphocytes were collected from the HA transgenic mice at two, five, and eight days after APC injections. The percentage of HA-specific $CD4^+$ T cells in the total $CD4^+$ T cell population of the mice was determined.

7. The percentage of HA-specific $CD4^+$ T cells in the mouse injected with APCs specific for HA-specific T cells was greatly reduced two days following injection compared to the mouse injected with the control APCs or untreated control mice. Specifically, the percentage of HA-specific $CD4^+$ T cells was sharply reduced to 9.4% compared to 59.2% in the mouse injected with control APCs, or 48.8% in the untreated control mice. At 5 and 8 days post APC injection the percentage of HA-specific $CD4^+$ T cells rebounded somewhat but remained reduced compared to the mouse that received the control APCs or the untreated control mice. See Table 1.

Table 1

APCs injected into mouse that express	% $CD4^+$ T cells expressing the HA receptor at 2 days	% $CD4^+$ T cells expressing the HA receptor at 5 days	% $CD4^+$ T cells expressing the HA receptor at 8 days
HA-LAMP-sig + FasL +trFADD	9.4	20.9	24.5
FasL + FADD	59.2	40.8	39.1
No APCs injected	48.8	48.8	48.8

8. Proliferation of HA-specific T cells in the transgenic mice was also assessed. The mice were euthanized twelve days after injection. Lymph node cells and splenocytes were obtained from the euthanized mice and were stimulated with HA *in vitro*. Proliferation was measured by determining the amount of radioactivity incorporated into cells one day after pulsing with ^{3}H-TdR (tritiated deoxyribothymidine).

9. We found that proliferation of HA-specific splenocytes was reduced by 3.0-3.6 fold in

the HA transgenic mouse injected with APCs specific for HA-specific T cells compared to the mouse that received the control APCs or the two untreated control mice. Similarly, proliferation of HA-specific lymph node cells was approximately 2.4-2.7 fold lower in the HA transgenic mouse injected with APCs specific for HA specific T cells compared to the transgenic mouse injected with control APCs or two untreated control HA transgenic mice. See Table 2.

Table 2

APCs that express	Counts measured in splenocytes ($x10^3$)	Counts measured in lymph node cells ($x10^3$)
HA-LAMP-sig + FasL +trFADD	34.8	48.6
FasL + FADD	106.5	129.9
No APCs injected	125.9	118.3

10. The flow cytometry and cell proliferation measurements are consistent with and support the claimed method of using APCs specific for a particular antigen-specific T cell population to activate those particular antigen-specific T cells. Co-administration of a product which is detrimental to activated T cell proliferation leads to a reduction in the antigen-specific $CD4^+$ T cell population and leads to a reduction in antigen-stimulatable proliferation of splenocytes and lymphocytes.

11. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these were made with the knowledge that false statements made willfully are punishable by fine, imprisonment, or both a fine and imprisonment under Section 1001 of Title 18 of the United States; and further that false statements made willfully may jeopardize the validity of any patent issuing on an application in which the false statements were made.

1/3/02
Date

Daniel B. Drachman